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The Locus of Cell Mechanisms

Terra Incognita between Cytology and Biochemistry

Until these ‘accidents’ occurred, workers engaged in the exploration of living organisms had been forced to stop at the edge of a mysterious no-man’s-land, bounded at the upper level of the dimension scale by the resolving power of the light microscope, and at the lower level by the applicability of chemical techniques. They knew, in a frustrating sort of way, that the area between these two boundaries contained some of the essential clues without which life would remain forever ununderstandable. With the technical advances mentioned, this region suddenly became accessible, both to visual examination right down to the level of macromolecules, and to chemical separation and analysis right up to the level of microscopic entities.

(de Duve, 1963–4, pp. 49–50)

Having described in abstract terms what mechanisms are and how they figure in scientific explanation, I turn now to setting the stage historically for the contributions of cell biology. The project of identifying cell mechanisms began in earnest after 1940 in what was then unoccupied territory between cytology and biochemistry. Researchers were at best dimly aware that crucial cellular operations occurred in organelles for which no direct methods of investigation were available. These organelles were too small to be meaningfully examined with the light microscope and much larger than the reacting molecules in homogenates that biochemists prepared from broken cells. Investigating these organelles as mechanisms required structural tools more powerful than those of cytology, functional tools building on those already developed in biochemistry, and new techniques incorporating both types of tools in order to integrate structure and function.

Christian de Duve, in the quotation above, refers to the “no-man’s-land” in which these mechanisms resided.¹ To discover them, researchers needed new instruments and strategies of investigation. Those will be the focus of the next chapter. Nonetheless, cell biologists utilized knowledge obtained in both cytology and biochemistry in their investigations. Thus, before turning to cell biology proper, I need to consider both what cytology and biochemistry could provide as well as their limitations when it came to the activities of cell life. I will pursue this project historically, examining, albeit in abbreviated fashion, how both cytology and biochemistry reached the state they had by 1940.

The differences between cytology and biochemistry involved not just the size of the objects of their study. Both fields were engaged in decomposing living systems, but cytology emphasized the structural decomposition of tissues, first into cells and then into organelles, while biochemistry emphasized functional decomposition of metabolic activities down to individual biochemical reactions.

1. CYTOLOGICAL CONTRIBUTIONS TO DISCOVERING CELL MECHANISMS UP TO 1940

Because cells are generally too small to be seen by the naked eye, their discovery depended on the development of what was in the seventeenth century a new tool – the microscope. The origins of the microscope are obscure but likely involved someone inserting a lens into a viewing tube, which would provide an approximately tenfold increase in magnification. Anton van Leeuwenhoek became familiar with the use of lenses to magnify cloth to count its threads when he was an apprentice in a dry goods store in Holland. He established new methods for grinding and polishing lenses and made a single-lens microscope that magnified objects up to 270 times. Using this instrument, van Leeuwenhoek identified what he termed *animalcules* in blood, sperm, and water from marshes and ponds. In the same period Robert Hooke² developed a compound

¹ de Duve (1984, p. 11) again used the metaphor of a no-man’s-land and referred to the region as *terra incognita* in characterizing knowledge of cells at the beginning of the 1940s: “there remained between the smallest entity discernible in the light microscope and the largest molecular size accessible to chemistry, an unexplored no-man’s-land extending over two orders of magnitude, a vast region that had to be labeled terra incognita on the map of the living cell.”

² Other investigators of the period who reported looking at plant and, sometimes, animal tissue with microscopes, included Nehemiah Grew, Marcello Malpighi, and Jan Swammerdam. See Hughes (1959) for a more detailed account of this early history. Gall (1996) presented pictures of several early microscopes as well as investigators’ drawings of what they saw.

microscope, albeit one that provided less resolution than van Leeuwenhoek's single lens. Hooke published many drawings from his microscopic observations of such biological objects as insects, sponges, bryozoans, foraminifera, and bird feathers in his 1665 book *Micrographia*. The most celebrated image in the book is a drawing of cork, in which he identified small cavities that he labeled *cells*³ (see Figure 3.1).

I took a good clear piece of cork, and with a pen-knife sharpened as keen as a razor, I cut off . . . an exceeding thin piece of it, and placing it on a black object plate, cause it was itself a white body, and casting the light on it with a *plano-convex glass*, I could exceedingly plainly perceive it to be all perforated and porous, much like a honey-comb, but that the pores of it were not regular; yet it was not unlike a honey-comb in these particulars. First, in that it had a very little solid substance, in comparison of the empty cavity that was contained between . . . Next, in that these pores, or cells, were not very deep, but consisted of a great many little boxes, separated out of one continued long pore . . . (Hooke, 1665, pp. 112–13)

Two types of artifacts produced by simple lenses seriously hampered advance in microscopic observation for two centuries. Spherical aberration resulted from the fact that light rays that leave a lens at different distances from the axis come into focus at different points. Chromatic aberration resulted from the fact that when light passes through a lens, the component wavelengths refract to different degrees. These aberrations often resulted in misleading observations, especially when their effects were compounded by use of a multi-lens system. At the beginning of the nineteenth century researchers such as Henri Milne Edwards and René Joachim Henri Dutrochet spoke of seeing *globules*, which they construed as the building blocks of organisms.⁴

³ The Latin term *cella* designates a small room (and was applied, for example, to the small memorial chapels erected in cemeteries). The choice of the term reflects the fact that for Hooke, focusing on plants, it was the walls surrounding a cavity that made for cells. E. B. Wilson commented on the adoption of the name: “The term ‘cell’ is a biological misnomer; for whatever the living cell is, it is not, as the word implies, a hollow chamber surrounded by solid walls. The term is merely an historical survival of a word casually employed by the botanists of the seventeenth century to designate the cells of certain plant-tissues which, when viewed in section, give somewhat the appearance of a honeycomb.” Wilson went on to say with respect to the conception of cell that had emerged by the end of the nineteenth century: “Nothing could be less appropriate than to call such a body a ‘cell’” (Wilson, 1896, pp. 13–14).

⁴ Reference to *globules* as the units in animal tissue goes back at least to Leeuwenhoek. Unlike plants, the units in animals lacked walls and thus the term *cell* was not regarded as appropriate. One of the tip-offs to the fact that these observers were seeing artifacts, not actual cells, was their insistence that the units were of uniform size. Milne-Edwards contended that all animal tissue is comprised of “a rather large number of these corpuscles which might differ in their constitution,

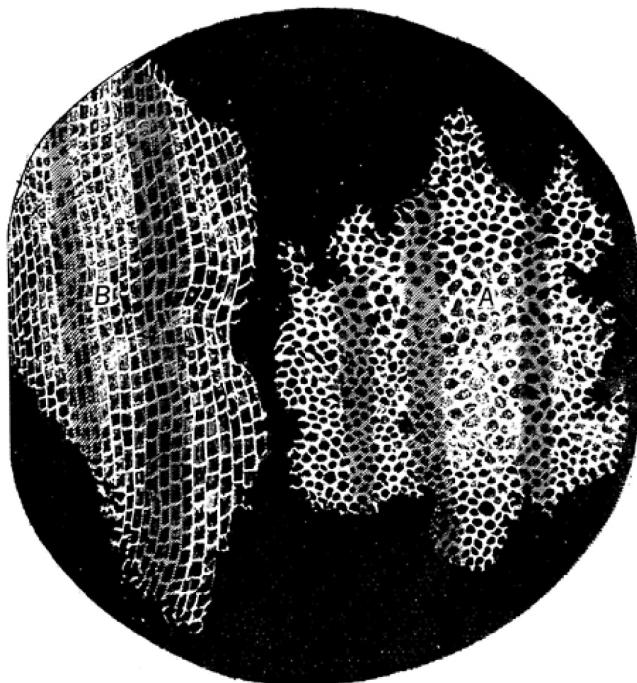


Figure 3.1. Robert Hooke's drawing of his observations of cork. He identified the small bounded areas as cells and noted in particular that the cells on the left side (labeled B) appeared to be boxes. Reproduced from R. Hooke (1665), *Micrographia: or some physiological descriptions of minute bodies made by magnifying glasses with observations and inquiries thereupon*. London: John Martin and James Allestry, Plate 11, Figure 1.

Sometimes these investigators may actually have been observing cells, but all too often what they observed were merely artifacts of their instruments.

Beginning in the late eighteenth century, a number of lens makers, including John Dollond, Joseph Lister, and Giovanni Amici, devised techniques using combinations of different glasses that greatly reduced chromatic aberration and also corrected for spherical aberrations. Microscopes using these *achromatic lenses* became available in the 1820s and 1830s.⁵ (Despite their

but which vary but little in their shape and probably in their volume" (1823, translated in Harris, 1999, p. 176). He specified that they were 1/300 mm in diameter.

⁵ Friedrich Gustav Jacob Henle, himself a major contributor to microscopical anatomy and histology, described the introduction of new microscopes to Müller's laboratory where he and Schwann were working: "Those were then happy days which the present generation might well envy us, when one saw the appearance of the first good microscopes from the firms of Ploessl at Vienna and from Pistor and Schlieck at Berlin, which we students bought with what money we were able to save" (Frédéricq, 1884, p. 13, as quoted in Hughes, 1959, p. 10).

name, they did not completely eliminate chromatic aberrations. In 1886 the collaboration of Ernst Abbe, Otto Schott, and Carl Zeiss resulted in the introduction of *apochromatic lenses*, which substantially reduced these residual chromatic aberrations.) The procedure for viewing something under these early microscopes was rather direct and straightforward: “With most animal tissues the usual practice was to tease out or squash fresh material into a layer of sufficient thinness, and to study this directly under the microscope” (Hughes, 1959, p. 13). With the dramatic improvements in microscopes, even such crude methods of specimen preparation were sufficient for knowledge of cells to burgeon during the nineteenth century.

Cytology in the Nineteenth Century

Numerous investigators made significant contributions to cytology as early as the first half of the nineteenth century including, for example, Johann Evangelista Purkinje and his student Gabriel Gustav Valentin, who made influential observations of neurons. However, it was Matthias Schleiden and Theodor Schwann who played the pivotal role by advancing what came to be known as the *cell doctrine* or *cell theory*. This unifying doctrine held that cells are the fundamental structural and functional units of life – adequate to comprise single-celled organisms but also serving as the building blocks of multicelled organisms. Ironically, many of the features of their accounts of cells that helped secure the initial acceptance of the cell doctrine proved false.

Schleiden’s research directly built on the finding by Robert Brown (1833), in his microscopic investigations of orchids, of the ubiquitous presence of “a single circular areola, generally somewhat more opaque than the membrane of the cell” (p. 710).⁶ He found one such structure in each cell and named it the *nucleus*. Schleiden, referring to it as the *cytoblast*, construed the nucleus as the most important region of the cell and that from which the rest developed. Growth from a nucleus became, for him, the characteristic feature through which he identified cells in different tissues. Schleiden was a botanist and initially limited his statement of the cell doctrine to plants.⁷ The challenge

⁶ Similar structures had been described previously in some animal tissues, e.g., in the epithelial cells by Felice Fontana in the 1780s and in the stigma of *Bletia Tankervilleae* by Franz Bauer in the 1790s.

⁷ In *Beiträge zur phytogenesis*, Schleiden construed whole animals as individuals but claimed that plants are formed of “fully individualized, independent, separate beings” – the cells (Schleiden, 1838). In Schleiden (1842) he mentioned approvingly, if tentatively, Schwann’s extension of the cell doctrine to animals.

facing microscopists examining animal tissues is that the cells of different tissues appear quite different. Moreover, these cells lack a cell wall, still treated by most investigators as the defining characteristic of plant cells. Schleiden's associate Schwann, however, focused on the similarities between the constituent units of different animal tissues and plant cells.⁸ The most important similarity was the nucleus, which Schleiden had shown him in plant cells. Finding opaque spots in the units of different animal tissues, especially those in an embryological state, Schwann identified them as nuclei. Because all animal tissue he examined contained nuclei, at least early in development, Schwann concluded that all animal tissues were comprised of cells despite their varied appearance. Figure 3.2 presents Schwann's drawings of several different cell types with their nuclei.

At the heart of both Schleiden's and Schwann's characterization of cells was an account of their formation. Schleiden (1838) had proposed that cells formed within preexisting cells through a physical process of accretion, first of the material comprising the nucleus around the nucleolus, and then another layer, corresponding to the cytoplasm, around the nucleus.⁹ Schwann (1839/1947) adopted this mechanism for animal cells, but situated the process not in existing cells but in the intercellular fluids. As a result, Schwann referred to the process in animals as *exogenous cell formation* and that in plants as *endogenous cell formation*. This account of cell development provided the foundation to the cell doctrine: “The elementary parts of all tissues are formed of cells in an analogous, though very diversified manner, so that it may be asserted that there is one universal principle of development for the elementary parts of organisms, however different, and that this principle is the formation of cells” (p. 165).

Given that other investigators were already describing cell division, and that Schleiden and Schwann were masters of microscopic technique, a question arises as to why they were convinced that cells formed in such a manner. Here it is important to appreciate that a central objective for Schwann in developing his cell theory was to provide a mechanistic account of the basic structures of living organisms. (Schwann was a member of the group of investigators working with Johannes Müller who pursued such a mechanistic vision

⁸ Schwann was working in the laboratory of Johannes Müller, who had himself observed cells in the *chorda dorsalis* and noted the similarity between them and plants (Müller, 1835) and had drawn Schwann's attention to the similarities.

⁹ One attractive feature for Schleiden of his account of cell formation was that it provided a role in development for the nucleus. Noting its regular appearance in embryos, Schleiden had made accounting for its role in development a major objective.

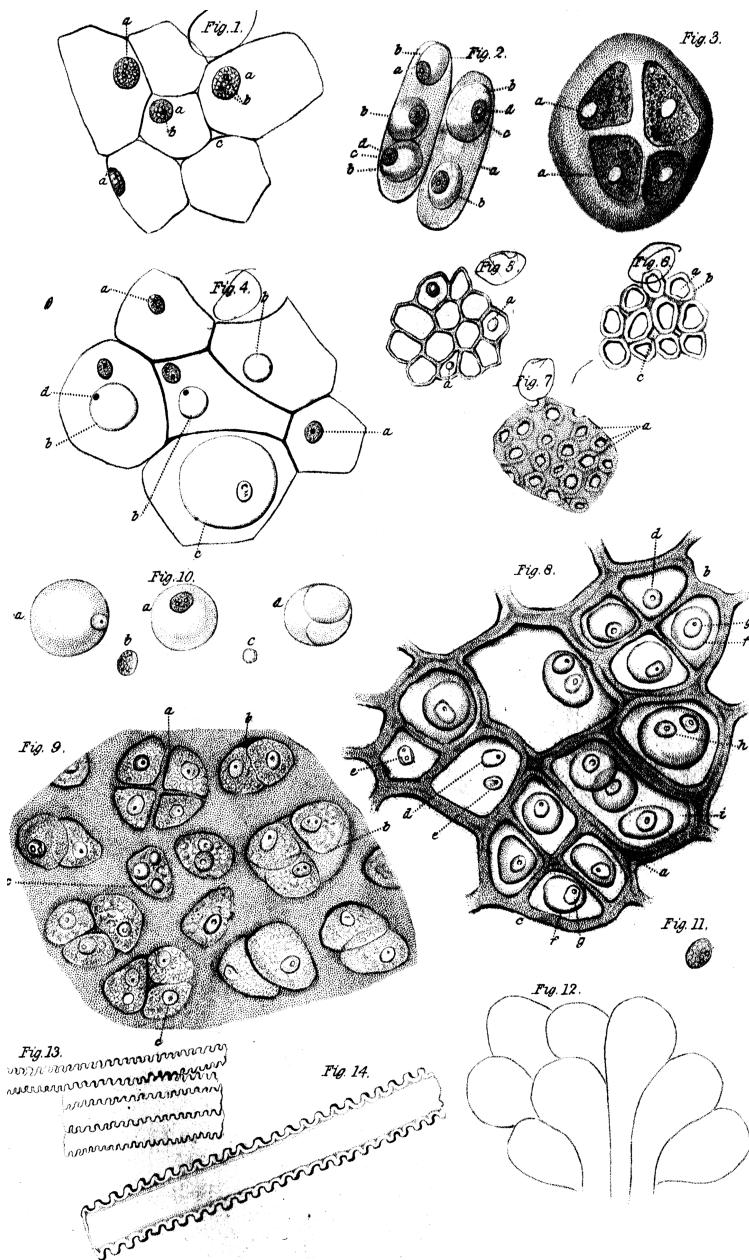


Figure 3.2. Schwann's drawings of different cell types. The first three figures are of plant cells – onion parenchymatous cellular tissue (1) and *Rhipsalis salicornoides* pollen (2 and 3, supplied by Schleiden), *Cyprinus erythrophthalmus* (a ray-finned fish) chorda dorsalis (4) and cartilage (5–7), *Rana esculenta* (frog) cartilage (8), *Pelobates fuscus* (toad) cranial cartilage (9), fetal pig crystalline lens (10–12), pike lens (13), and grass epidermis (14). Reproduced from Plate I from T. Schwann (1839/1847), *Microscopical researches into the accordance in the structure and growth of animals and plants* (H. Smith, trans.). London: Sydenham Society.

throughout physiology.)¹⁰ Schwann drew a parallel between cell formation and crystal formation. Even though the details of crystal formation were not yet known, it was clearly a mechanical process not requiring any vital forces, and this made it a very compelling model for Schwann. He proposed that a mechanism of attraction drew a particular kind of material out of the inter-cellular fluid, with each layer (the nucleolus, the nucleus, and the cytoplasm) drawing out different substances. He discounted the importance of differences between inorganic crystals and biological cells: “If crystals were formed from the same substances as cells, they would probably, in these respects, be subject to the same condition as cells” (Schwann, 1839/1947, p. 208; for further analysis, see Bechtel, 1984).

Schwann’s commitment to chemical mechanisms for explaining vital phenomena was already manifest in his earlier research on digestion, in which he discovered pepsin, the first enzyme identified in animals (Schwann, 1836). In the eyes of some mechanists, Schwann’s commitment to mechanism was compromised when, in 1837, he presented evidence that fermentation was a process requiring living yeast cells (Schwann, 1837). Many mechanists associated such a claim with vitalism. But Schwann did not see himself as embracing vitalism and in part III of *Microscopical Researches*, the book in which he introduced his cell theory, he returned to the topic of fermentation, presenting it as an example of the kind of metabolic phenomena exhibited in cells (Schwann here coined the term *metabolism*).¹¹ He proposed that the ability of living cells to carry out activities that would not otherwise occur in nature was due to the distinctive chemical constitution of cells that resulted from the process of cell formation, itself a mechanical process. The significance of Schwann’s contribution was to fix the cell as the locus of control (Bechtel & Richardson, 1993, Chapter 3) of basic life functions such as nutrition.¹² He

¹⁰ Müller’s own position regarding vitalism was more ambiguous, but among his students many, such as Emil du Bois-Reymond, Ernst von Brücke, Hermann von Helmholtz, and Carl Ludwig, were outspoken advocates of a mechanistic physiology (see Cranfield, 1957).

¹¹ Schwann characterized a cell as having “the faculty of producing chemical changes in its constituent molecules. Besides which, all the parts of the cell itself may be chemically altered during the process of its vegetation. The underlying cause of all these phenomena, which we comprise under the term metabolic phenomena, we will denominate the *metabolic power*” (Schwann, 1839/1947, p. 197).

¹² Schwann’s strategy basically was to argue that whatever activities cells perform would not be performed twice, once in the cell, then again in the whole organism: “Now, as all cells grow according to the same laws, and consequently the cause of growth cannot in one case lie in the cell, and in another in the whole organism; and since it may be further proved that some cells, which do not differ from the rest in their mode of growth, are developed independently, we must ascribe to all cells an independent vitality, that is, such combinations of molecules as occur in any single cell, are capable of setting free the power by which it is enabled to take up fresh

also pointed the way to further developing a mechanistic account of cellular functions in terms of the component parts found in cells and their operations, but was not in position to develop a detailed account himself; that task was left for later scientists.

Although Schwann's contention that cells were the basic building blocks of all organisms was widely accepted, his and Schleiden's mechanism for cell formation became the focus of controversy. Researchers such as Barthélemy Dumortier (1832) and Hugo von Mohl (1837) reported observations of dividing cells in *Confervula aurea* even before Schleiden's publication,¹³ and von Mohl as well as Franz Unger and Carl von Nageli continued in subsequent years to develop ever more detailed descriptions of cell division. These included observations that the division of the nucleus preceded the division of the cell itself. Even von Mohl, though, allowed that sometimes cells formed like crystals and for a number of years the two accounts, cell division and Schleiden's and Schwann's accounts, were considered to provide two ways in which new cells were created. Rudolf Virchow (1858), a pathologist, and Robert Remak (1852; 1855) provided what turned out to be the decisive argument against Schwann's account – it amounted to spontaneous generation.¹⁴ Virchow argued instead that each cell arises from a pre-existing cell (*omnis cellula e cellula*).¹⁵ Virchow could offer no specific mechanism for cell division, but he was not as committed to mechanism as Schleiden and Schwann. He viewed the difference between healthy and pathological life as involving organizational properties of whole cells. Accordingly, he characterized his approach to pathology as “cellular pathology” (Virchow, 1855) and appealed

molecules. The cause of nutrition and growth resides not in the organism as a whole, but in the separate elementary parts – the cells” (Schwann, 1839/1947, p. 192).

¹³ Schleiden actually cited von Mohl's paper, but contended that von Mohl was deceived by the smallness and transparency of the new cells that had formed in the old ones and so was led to see the final breaking free of these new (and already fully developed) cells from the mother cell as a process of cell division.

¹⁴ Remak (1852) commented, “As for myself, the extracellular formation of animal cells struck me, from the very moment that this theory was propagated, as no less improbable than the generation aequivoqua of organisms” (translated by Henry Harris (1999, p. 130). There is irony in the contention that Schwann's theory of cell formation amounted to spontaneous generation because in his paper on fermentation he had come down against spontaneous generation by demonstrating that fermentation required a microorganism that was killed by heat (Schwann, 1837).

¹⁵ The expression was not original with Virchow. François-Vincent Raspail (1825) used it as an epigraph. When Virchow first employed the phrase, he wrote it “omnis cellula a cellula,” a more ambiguous expression asserting only that all cells arise by means of cells rather than explicitly deriving from them.

to a “life force” to account for the specific activities that occur in living cells (this life force being acquired from other cells).

Although the cell wall had been the focus of investigations for plant cells from the time of Hooke through the investigations of Brown and Schleiden, Hooke had already noted the fluid contents of cells. He labeled them “*succus nutritus*, or appropriate juices of vegetables,” and described cells “fill’d with juices, and by degrees sweating them out” (Hooke, 1665, p. 116). With the improved microscopes of the 1830s, featuring achromatic lenses, more investigators began commenting on the fluid contents of cells. Von Mohl, for example, described “an opaque, viscid fluid, having granules intermingled in it” as a universal feature of cells (1852, p. 37). In 1846 von Mohl had applied the term *protoplasm*¹⁶ to the fluid. Slightly earlier, Dujardin (1835) labeled the viscid, slimy fluid found in animal tissues *sarcode*.¹⁷ Remak (1852) pointed to the similarities between plant protoplasm and animal sarcode and employed the term *protoplasm* for both. This identification was cemented when Max Schultze (1861) characterized a cell as “a lump of protoplasm inside of which lies a nucleus” (p. 11). For Schultze, protoplasm was sufficiently nonmiscible with fluids surrounding the cell that a cell membrane was not needed; he rather viewed it as a sign of cell senility.

As I will discuss below, cytologists for the most part kept their focus on the structural components of the cell and on the processes involved in cell division. A number of chemically minded physiologists, though, made the special chemical nature of protoplasm their pursuit. Many of them also developed mechanistic accounts of cell functioning. One exemplar of this approach was T. H. Huxley’s popular essay, “On the physical basis of life” (Huxley, 1869), in which he proposed a three-point unity among living things – they all exhibit the power of contractility, are composed of cells (defined à la Schultze), and are made of protoplasm. He took protoplasm to be comprised of proteins, whose chemical composition of carbon, hydrogen, nitrogen, and oxygen had been established in the 1830s. Huxley’s objective was simply to provide a

¹⁶ Six years earlier Purkinje introduced the same term for the embryonic material in animals, but von Mohl seems to have been unaware of this. Part of the significance of protoplasm for von Mohl was that he viewed it as providing the material for a new cell nucleus, whose formation would prompt division of the old cell.

¹⁷ Dujardin made it clear that he was giving a name to a substance that had been observed earlier by others: “I propose to give this name to what other observers have called a living jelly, this glutinous, diaphanous substance, insoluble in water, that contracts into globular lumps, sticks to dissecting needles, and can be drawn out like mucus. It is to be found in all lower animals interposed between other structural elements” (1835, translated by Harris, 1999, p. 74).

physical grounding to biological inquiry, but other theorists were interested in the apparently special powers of protoplasm in metabolic processes. I will discuss some of the most prominent chemical accounts of protoplasm in the second part of this chapter.

Returning to cytology, the second half of the nineteenth century was a period of very active investigation. The technique of microscopy underwent significant changes in the decades after 1850. I have already noted the introduction of apochromatic lenses, which further reduced chromatic aberrations. Although chemical reagents such as chromic acid had sometimes been applied as preservatives and “hardening agents,” a major step was the discovery that using osmic acid as a fixative would preserve the fine detail of cells (although it also gave rise to the question of whether it revealed existing structure or generated artifacts). Schultze (1865) pioneered this approach in his study of the luminescent organ of the glowworm. Fixation served both to kill the cells in the tissue and to stabilize the structure that would otherwise be disrupted postmortem by processes of *autolysis*. Embedding in hard materials was important because it made it possible to cut thin slices of material through a process known as *sectioning*. The contents of a given section could then be viewed without being occluded by the contents of other sections.

Equally important was the development of stains that would selectively color different components of cells. Carmine red was the first stain to be employed in this way, by Alfonso Corti and Joseph von Gerlach in the 1850s. (Produced from the crushed and dried bodies of cochineal insects in Mexico, it had been used there as a dye for centuries.) Gerlach found that the nucleus selectively absorbed carmine, resulting in a far clearer image of it than had been available previously. In the following decade Böhmer applied hematoxylin, obtained from logwood, a tropical American tree, to stain the nucleus deep blue. In the 1850s William Perkin discovered aniline dyes while he was trying to synthesize quinine, and in the 1860s–70s other investigators determined that some aniline dyes would stain parts of the cell not affected by carmine or hematoxylin. This made it possible to stain different structures in different colors.

The greatest advances in understanding cell mechanisms in the second half of the nineteenth century involved the events of cell division. As I noted previously, Brown gave the nucleus its name and Schleiden proposed a central role for it in his account of cell formation. As accounts in terms of division of existing cells supplanted Schleiden’s and Schwann’s accounts of crystal-like cell formation, there was considerable uncertainty about the role of the nucleus. Karl Bogislaus Reichert (1847) contended that the nucleus disappeared when cells divided and that new nuclei were created in daughter cells.

In opposition, Remak (1852) contended that the nucleus itself divided prior to cell division but noted, “Observations on the mechanisms of nuclear division are by no means so extensive as those on the behaviour of the cell membranes” (translated by Harris, 1999, p. 139). Three years later he contended that nuclear division was preceded by division of the nucleolus through a process of constriction and nuclear membrane partitioning analogous to that found in division of the cell body (Remak, 1855).

One of the first microscopists to contend that the division of the nucleus was more complex than a simple division was Wilhelm Hofmeister (1849). He observed in pollen mother and staminal hair cells of *Tradescantia*, a plant used for ground cover, that the nuclear membrane dissolved before the cell divided. If he stained the cell with iodine, however, he could observe discrete lumps (*Klumpen*, presumably chromosomes) that Hofmeister proposed were protein coagulates. He reported that a *granular mucilage* formed around these lumps and divided into two, with a membrane forming around each as the cell divided.

The availability of fixatives, stains, and finally apochromatic lenses enabled research on nuclear processes in cell division to explode in the 1870s and 1880s. Edouard van Beneden (1875) characterized the structures in the nucleus as little rods (*bâtonnets*) and observed that they moved apart in the process of nuclear division. Working with fertilized eggs of the marine mollusk *Geryonia*, Hermann Fol (1873) described the configuration of the spindle and astral rays and proposed an analogy with the lines of force found between opposite magnetic poles, suggesting thereby a dynamical perspective. Focusing on cell division in conifer embryos, Eduard Strasburger, in the first edition of his *Zellbildung und Zelltheilung*, presented drawings portraying a fibrous spindle at several stages of division.

Walther Flemming (1878; 1879) devoted considerable effort to developing new fixatives that would better reveal the details of the process of nuclear division. The product was a mixture of chromic, osmic, and glacial acetic acids that came to be known as Flemming’s solution. Because the rods responded to this and other stains, Flemming referred to them as *chromatin*, contrasting them with unstained structures he labeled *achromatin*. (Waldeyer, 1888, gave them their current name, chromosomes.) Working with salamander larval epithelial cells, Flemming observed that the rods split lengthwise and proposed that one half went to each of the two daughter cells. To distinguish this far more complex sequence of events from the simple form of nuclear division described by Remak, Flemming referred to the process as “indirect nuclear division” and introduced the term “*Karyomitose*” for the changes in the chromatin. Flemming reviewed his findings and presented several detailed figures of the

process of mitosis in his 1882 book *Zellsubstanz, Kern und Zelltheilung*. In an obvious play on Virchow's dictum, he invoked the dictum *Omnis nucleus e nucleo* for his account.

In addition to this research on mitosis, numerous researchers observed the processes of fertilization and began to propose accounts of the continuity of chromosomes from parents to offspring. Strasburger (1884) described the chromosomes of the offspring as originating half from the father and half from the mother. Van Beneden (van Beneden & Neyt, 1887) discovered that prior to fertilization the chromosomes in both the egg and sperm nuclei were reduced in half (the process is known as *meiosis*) and that a full complement of chromosomes only reappeared after fertilization of the egg. Van Beneden was able to sustain this claim by showing that what he termed pronuclei in the nematode (*Ascaris maglocephala*) possessed only two *anses chromatiques* rather than the typical four. Flemming (1887) then described the process of reducing the number of chromosomes in sperm production.

This line of research on mitosis and meiosis represented the major nineteenth-century success stories in describing mechanisms of cell life. Although some cytologists were simply interested in identifying stages in the overall operation of splitting cells, others were keenly interested in the potential of these operations to explain heredity. August Weismann (1885) drew attention to the fact that the procedures of cell division insured that daughter cells received one complete complement of the hereditary material by drawing half from each parent. Thus, when Carl Correns (1900) participated in the rediscovery of Mendel's work, he made explicit the connection between the steps in meiosis and Mendel's account of inheritance: Each germ cell must receive one or the other of the factors Mendel held responsible for the dominant or recessive traits. The relation between chromosomes and Mendel's factors, rechristened *genes*, was developed in much greater detail in the first half of the twentieth century by researchers of the Morgan school, who discovered such additional phenomena as the crossing over of chromosomes during meiosis.

Thus, one effect of the introduction of fixatives and stains was an initial understanding of mechanisms of nuclear division in the late nineteenth century that laid a foundation for genetic accounts of the twentieth century. Fixatives and stains also sparked observations and theorizing about structures and operations in the cytoplasm. These proved far more contentious. For example, Leydig, Carnoy, and Heidenhain claimed to identify structures they called *fibrils*, which they proposed accounted for the unusual consistency of protoplasm. Hanstein identified particles he called *microsomes* embedded in these fibrils. Critics such as Flemming, Bütschli, and Fischer charged that the fibrils were artifacts. They introduced what became a common

argumentative strategy – demonstrations that they could artificially produce structures with the same appearances as fibrils by, for example, coagulating the whites of eggs or gelatin with various fixatives. I will turn to the question of how scientists appraise claims about artifacts in the next chapter.

The pattern of claim and counterclaim concerning the reality of various proposed cytoplasmic constituents continued through the early years of the twentieth century. This period also witnessed further improvement in the light microscope and the techniques for using it, as well as the introduction of variations, such as the ultraviolet microscope and phase contrast microscope. Moreover, as is clear from a 1924 textbook edited by Edmund Cowdry, *General cytology: A textbook of cellular structure and function for students of biology and medicine*, it was a period in which cytologists were increasingly interested in figuring out the function of new structures and making connections to chemistry (though often frustrated by the limitations of available tools). In the following sections I describe in more detail what was being learned about cell membranes and three organelles – mitochondria, the ergastoplasm, and the Golgi apparatus – that would later become foci of inquiry using improved tools in the early cell biology of the 1940s and 1950s.

Cell Membranes (1825–1935)

Membranes are among the most important parts of cells, serving to organize other parts and functions. Not only is there a plasma membrane surrounding the cell itself, but also membranes enclose the cell organelles, including the nucleus. Although membrane boundaries can be readily seen in electron micrographs, they were not so easily distinguished with the light microscope. Much of the evidence for them in the nineteenth century stemmed from their role in selectively admitting substances into cells. Already in the late eighteenth century William Hewson (1773) had appealed to the ability of red blood cells to shrink or swell to argue that they were envelope-bounded vesicles. Hewson's research was not well known in the nineteenth century, however, and the presence of a barrier surrounding animal cells continued to be debated. Schultze (1861), as I noted previously, characterized a cell as a lump of protoplasm which acquired a membrane only in its senility.

One of the key types of evidence pointing to the existence of membranes was the phenomenon of osmosis, the movement of water in and out of cells until the substances dissolved in it are distributed equally on the two sides. During the period when animal cells were known as *globules* and reports of their existence may have been artifacts of chromatic aberrations, Henri

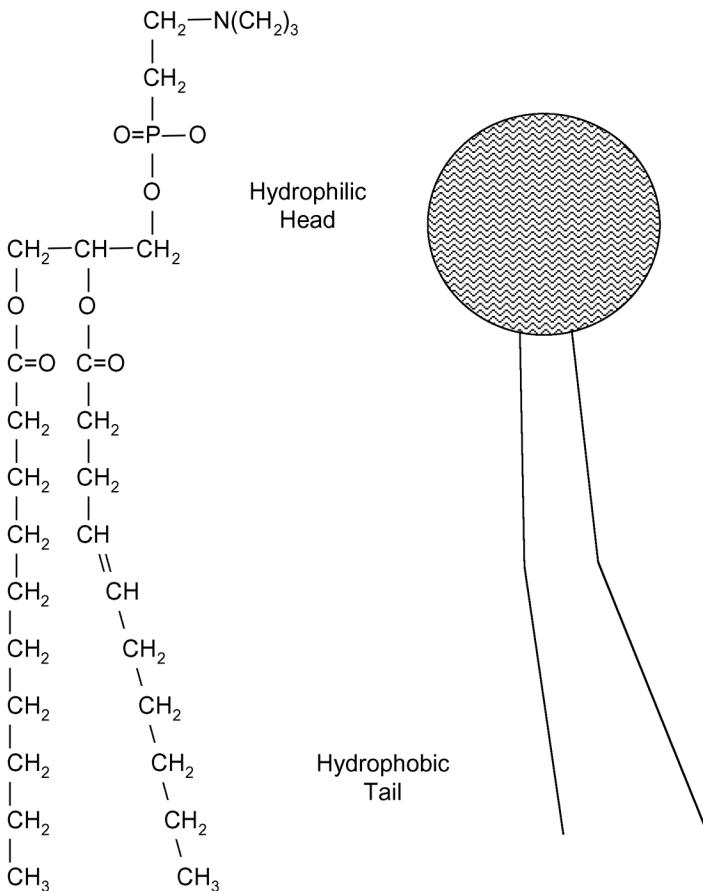


Figure 3.3. Structure of a typical phospholipid molecule with a hydrophilic head and hydrophobic tail.

Dutrochet (1826; 1828) characterized them as units of physiological exchange, selectively admitting nutrients along with the inflow of water, a process he termed *endosmosis*, and excreting waste with the diffusion out, which he termed *exosmosis*. Carl Wilhelm von Nägeli and Carl Cramer (1855) described osmosis in plant cells. Drawing on an analogy with osmometers that Moritz Traube had constructed using artificial semipermeable membranes, Wilhelm Pfeffer (1887) proposed that cells generally were surrounded by semipermeable membranes. Although early research on osmosis linked the ability of materials to cross membranes to their permeability in water, Charles Ernst Overton (1895; 1896; 1899) found in his attempts to get root hairs in plants to absorb substances that fat soluble substances passed through more

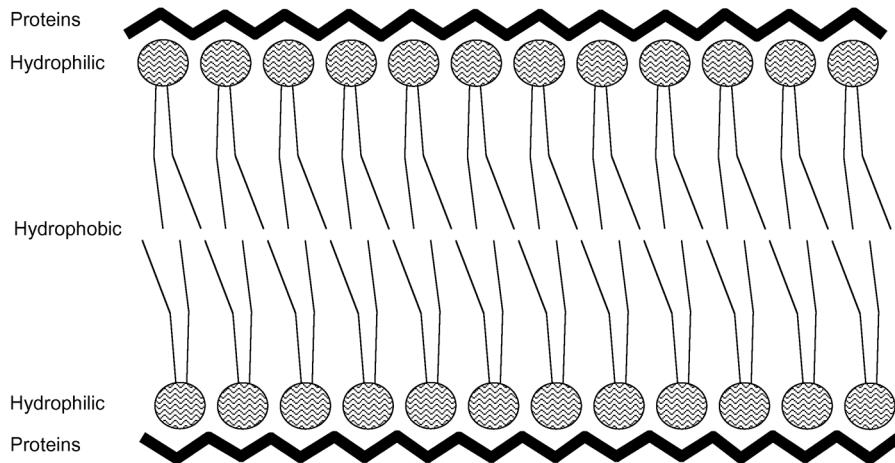


Figure 3.4. Danielli-Davson “sandwich” model of membrane structure. Following Gorter and Grendel (1925), phospholipids are arranged into a bilayer in which each molecule’s hydrophobic tail faces inward and hydrophilic head faces outward. Danielli and Davson (1935) added outer layers (jagged lines) composed of proteins.

easily than water soluble ones. Finding that membranes thus acted like fatty oils, Overton inferred that they were partly composed of lipids. Overton’s research served to settle the debate, establishing that membranes were a universal feature of cells.

Substantial advances in the understanding of membranes continued in the first half of the twentieth century. In 1925 Evert Gorter and Grendel extracted lipid from red blood cells and concluded that there was just enough for the cell to be “covered by a layer of fatty substances that is two molecules thick” (1925, p. 443). Each molecule was a phospholipid in which the head had an affinity for water (*hydrophilic*) and the tail did not (*hydrophobic*), as illustrated both by chemical formula and abstractly in Figure 3.3. Gorter and Grendel proposed that the molecules are arranged such that the heads of the two layers face out in opposite directions while the tails face inward. This would result in the outside of the membrane being hydrophilic but the inside hydrophobic. Fat soluble substances like hydrocarbons can pass through such a membrane, whereas water soluble substances such as sodium and potassium ions and sugars cannot.

In addition to lipids, investigators found proteins and carbohydrates in membranes. In 1935 James F. Danielli, a physical chemist, and Hugh Davson, a physiologist (Danielli & Davson, 1935), advanced an extremely influential sandwich model in which, as shown in Figure 3.4, a protein layer covers

each surface of a lipid bilayer like that proposed by Gorter and Grendel. Specifically, each protein is attached to the outward-facing head of one of the phospholipid molecules. This model offered a ready explanation for the appearance of membranes as pairs of dark lines with a lighter region between them in electron micrographs and was generally accepted for more than thirty-five years before being replaced by a model in which proteins were embedded in the membrane.

Mitochondria (1890–1925)

Discussions of mitochondria frequently attribute their discovery to the investigations of Richard Altmann around 1890.¹⁸ He introduced improved fixation techniques (e.g., a solution of potassium dichromate and osmium tetroxide). When used with an acid-fuchsin stain differentiated by picric acid, and with delicate heating, he was able to observe filaments in the cytoplasm of nearly all cell types. Although some of the structures Altmann saw were undoubtedly mitochondria, he positioned his discussion in a very different context than modern thinking about cell organelles. He titled his 1890 book *Die Elementarorganismen und ihre Beziehungen zu den Zellen* (*The Elementary Organisms and their Relations to the Cells*). His endeavor was to revise and revive the view that living substances were comprised of elementary living granules (the filaments were, for him, strings of granules). The revisions were designed to make the granular theory compatible with cell theory, and specifically with Virchow's dictum *omnis cellula e cellula*, which he then extended to granules with the dictum *omne granulum e granulo*.

In espousing the view that granules were the basic living unit, Altmann opposed the view that protoplasm has a uniform structure. He attributes basic metabolic processes (specifically, fat metabolism and secretion) to these granules. Moreover, he claimed granules were equivalent to independently existing microorganisms and named them both *bioblasts*: Because both granules and microorganisms “represent the elementary organisms which are found wherever vital forces become active we shall name them with the joint term Bioblasts. **It seems that with the bioblast [the] morphological unit of living matter has been found**” (Altmann, 1890, from an unpublished

¹⁸ This is not to say that Altmann was the first to see mitochondria. Starting around 1850 Kölliker studied granules in muscle cells. In 1888, he separated them from insect muscle and found that they swelled in water and possessed a membrane. These were probably mitochondria, but it is often very difficult to know for sure what structures (or artifacts) he and other early observers were actually observing.

translation prepared by Hanna Stoeckenius, folder 13, box 2, RU 518, Rockefeller University Archives, RAC, p. 125).

Many investigators responded to Altmann's reports of granules with great skepticism, raising doubts in particular about his reliance on fixatives such as osmic acid. The comments of William Bate Hardy are illustrative:

It is notorious that the various fixing reagents are coagulants of organic colloids, and that they produce precipitates which have a certain figure or structure. It can also readily be shown . . . that the figure varies, other things being equal, according to the reagent used. It is therefore cause for suspicion when one finds that particular structures which are indubitably present in preparations are only found in cells fixed with certain agents, used either alone, or in particular formulae. Altmann demonstrates his granules by the aid of an intensely acid and oxidizing mixture. (Hardy, 1899; quoted in Fruton, 1972, p. 389)

Fischer (1899) cast further doubt on the reality of the granules by showing that by applying commonly used fixing agents, especially osmium, to various homogeneous protein solutions (e.g., egg whites and gelatins, which would not contain subcellular structures, he could produce a variety of granular and filamentous structures. In part to counter charges of methodological artifact, Altmann also pioneered an alternative technique to chemical fixation, freeze-drying, which, while very laborious, provided an independent basis for evaluating the reality of the structures revealed by chemical fixation. This, however, did not suffice to stop the objections from the critics.

Carl Benda (1898; 1899), employing crystal violet as a stain, observed Altmann's structures and proposed the named *mitochondria*, from the Greek words for thread and granule. The name reflected the fact that in his preparations they sometimes appear threadlike and at other times more granular.¹⁹ Importantly, Benda provided evidence that these structures could be seen both in fixed and in living cells, reducing the plausibility of the objection that they were an artifact of fixation. Further, Michaelis (1899) showed that the dye Janus Green (diethylsafraninazodimethylanalin), which appears blue-green when oxidized but colorless when reduced, would turn mitochondria blue-green in living cells. This suggested that mitochondria had the capacity to oxidize substrates and provided the first clue as to their role in cellular respiration.

¹⁹ These different appearances, we now understand, depended upon the angle at which the mitochondrion was sliced in preparing the slide. The term *mitochondrion* only gradually became accepted. Some other terms were blepharoblast, condriokonts, chondriomites, chondrioplasts, chondriosomes, chondriospheres, fila, fuchsinophilic granules, interstitial bodies, Körner, Fädenkörner, mitogel, parabasal bodies, plasmasomes, plastochnidia, plastomes, spheroplasts, and vermicles.

With the tools available in the early twentieth century, researchers were able to develop two types of information about mitochondria: (a) their shape and location in cells, and (b) their composition. Microscopic examination showed that the appearance of mitochondria is relatively stable across species, but varies in degree of elongation and thickness according to the cell type. Cowdry (1924) noted that in gland and nerve cells as well as embryonic cells, they were observed as filaments but that on injury, their shape changes, “providing by far the most delicate criterion of many types of cell injury at our disposal” (p. 317).

Evidence about the composition of mitochondria came primarily from reactions with various reagents. Their solubility with acetic acid, as well as with alcohol, ether, and chloroform, indicated a phospholipid constitution. The failure of mitochondria to stain with Sudan III indicated that they did not contain fat, and failure to stain with Millon’s reagent indicated little if any protein.

Once dissociated from Altmann’s conception of an elementary organism carrying out all basic metabolic processes, a natural question was what function mitochondria perform. Kingsbury (1913) noticed that the fixatives yielding the best visualization of mitochondria – osmic acid, potassium dichromate, and formalin – all depend on reducing substances. Picking up the thread from Michaelis, he advanced the proposal that mitochondria play a critical role in respiration.²⁰ Cowdry further noted that the amount of mitochondria in a cell was positively correlated with its level of activity (division, secretion, etc.) and negatively correlated with the amount of fat in it (an indication of decreased respiration):

We have two lines of observation to harmonize: this association of abundant mitochondria with intense protoplasmic activity and a reciprocal relationship which appears to exist between the amount of mitochondria and the amount of fat. Where there are few mitochondria there is often much fat, and vice versa. Decreased oxidation favors the deposition of fat and increased oxidation hastens its elimination, which suggests at once the existence of some connection between the amount of mitochondria and the rate of oxidation; and their abundance in the more active stage of the life of the cell, when protoplasmic respiration is rapid, points to the same tentative conclusion. (Cowdry, 1924, p. 321)

²⁰ According to Bourne (1962, pp. 70–1), Kingsbury’s major evidence was that “anesthetics such as ether or chloroform, which depressed cellular respiration and the respiration of the animal in general, also broke up mitochondria in the cell.”

Even while reviewing this impressive evidence for the role of mitochondria in respiration, Edmund Cowdry himself was not convinced:

Although this view, that mitochondria take part in protoplasmic respiration, has been well received by cytologists and serves as a useful and convenient working hypothesis, it is still only a theory and must be regarded as such. (Cowdry, 1924, p. 325)

Cowdry preferred Claudius Regaud's (1909) interpretation that mitochondria served to select substances out of the cytoplasm and, after bringing them inside, condensed and transformed them into different products.

In the end, Cowdry was quite pessimistic about the prospects of rapid progress in the study of mitochondria:

it is quite obvious that the investigation of mitochondria will never achieve the usefulness which it deserves as an instrument for advance in biology and medicine until we know much more of their chemical constitution as the only accurate basis for interpretation of our findings. In other words, we must wait upon the slow development of direct, qualitative cellular chemistry. (Cowdry, 1924, p. 311)

Having made little more progress by the late 1930s, Cowdry, on the advice of Simon Flexner, Director of the Rockefeller Institute, abandoned the problem of mitochondria and moved to cancer research.

Ergastoplasm or Basophilia (1900–1930)

Investigators in the nineteenth century advanced a number of ideas about the constituency of the area of cytoplasm that largely appeared empty under the light microscope. One concept that would play a prominent role in the development of cell biology was that of *ergastoplasm*. According to Hague-nau (1958), it stemmed from the thesis research of Charles Garnier, a French cytologist in Nancy who explored the effects on cells of a number of stains, including safranin, gentian violet, and toluidine blue. He identified a fibrillar material or rod-like structure in gland cells. Because he thought this structure was associated with production of secretion granules, he called it *ergastoplasm*, that is, the plasma that elaborates or transforms something (Garnier, 1897; Garnier, 1900). Because the intensity of stain varied with the stage of secretion in gland cells, Garnier concluded that ergastoplasm was not a permanent structure but emerged during the resting phase after secretion. He also proposed a link between the ergastoplasm topographically and the nucleus,

claiming that at times the ergastoplasm formed around, and sometimes encircled, the nucleus. He suggested that the nuclear sap or chromatic substance from the nucleolus passed through the nuclear membrane and joined with the ergastoplasm. Garnier's proposal was further developed by Prenant (1898), the supervisor of Garnier's thesis, who termed the ergastoplasm *protoplasme supérieur* and characterized it as a zone of the cytoplasm that could differentiate into other structures.

As with the mitochondrion, investigators disagreed as to whether the ergastoplasm was a real structure. Regaud offered support for Garnier and Prenant, showing that when he added acetic acid to his fixative, the ergastoplasm appeared as Garnier reported but mitochondria were not visible, whereas if he left out the acetic acid, mitochondria appeared, but no ergastoplasm (Regaud, 1909). He also supported the idea that ergastoplasm involved cytoplasmic material which was "impregnated with chromatin or a closely related substance" (quoted in Haguenaau, 1958). In opposition, Morelle (1927) denied that the ergastoplasm had a fibrillar structure and proposed that it was simply modified ground cytoplasm which, due to its chemical composition, took up basic stains. Christian Champy (1911) proposed that ergastoplasm was simply poorly fixed mitochondria.

Although, as Haguenaau describes, there continued to be some publications describing the ergastoplasm, it largely faded from view in characterizations of cell structures in the first half of the twentieth century. It is not discussed, for example, either in Cowdry's *General cytology* (1924) or in Bourne's *Cytology and cell physiology* (1942).

The Golgi Apparatus (1900–1940)

What came to be known as the *Golgi apparatus* was first systematically observed in Purkinje and ventral horn cells of the barn owl and the cat by Camillo Golgi in 1898. He fixed his cells with an osmium-tetroxide-potassium dichromate mixture followed by impregnation with silver salts (Golgi, 1898). To Golgi it appeared as a fine network, which he characterized as an internal reticular apparatus (*apparato reticolare interno*; see Figure 3.5). A number of other investigators in the same period also described what was probably the same structure. Whaley provides an explanation as to why Golgi's work stands out:

Considering the fixatives and stains being used, the amount of experimental work with them characteristic of the latter part of the 19th century, and the multiplicity of the tissue and cell types being studied, it seems reasonable to suggest that a considerable number of investigators may have seen this pleiomorphic

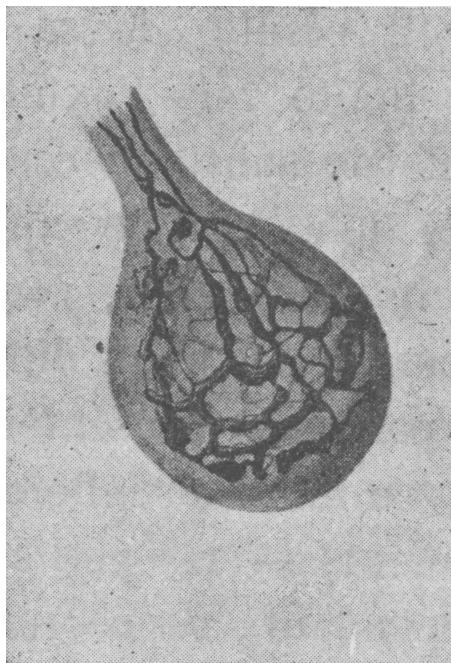


Figure 3.5. Golgi's drawing of what he identified as an internal reticular apparatus in a Purkinje cell of a barn owl. Reproduced from C. Golgi (1898), *Sur la structure des cellules nerveuses, Archives italiennes de Biologie*, 30, 60–71, Figure 2.

organelle before Golgi. It was Golgi, however, who devised a method that put the organelle in sharp contrast with other cellular components and permitted him and his students to demonstrate it as a consistent structural component in a wide variety of tissue cell types. It was also Golgi who recognized not only some of the details of its structure but also that it is variable in character and position. (1975, p. 3)

Shortly after Golgi's first publication, Emil Holmgren (1902) identified a set of clear canals within the cytoplasm that remained uncolored when the rest of the cytoplasm was stained. Holmgren construed these canals as formed by cytoplasmic processes penetrating into a cell from its neighbors and suggested that they might serve a nutritive function. He called them *trophospongium* and claimed they were the same as Golgi's reticular nets although they were located near the external membrane of the cell.

Initially Santiago Ramón y Cajal (1907; 1908) accepted the link between Golgi's reticular structure and Holmgren's canals and referred to them as the Golgi-Holmgren canals. Later he revised this assessment (Cajal, 1914)

and switched to Józef Nusbaum's term, *Golgi apparatus*.²¹ Cajal went on to examine how its appearance varied with different cellular functions and with different experimental manipulations. Without endorsing the claim that it figured in secretion, Cajal noted a correlation between the presence of the Golgi apparatus in a cell and secretory activity and reported, in intestinal goblet cells that secrete mucus over the lining of the intestine, the appearance of tiny droplets of mucus in the Golgi region.

Progress in clarifying the structure and determining the function of the Golgi apparatus was slow. In 1924, Cowdry commented,

Even now, twenty-five years after its discovery, we can only say that the Golgi apparatus is an area of the cytoplasm frequently (especially in higher forms) of reticular shape, often as large as the nucleus, and sometimes definitely located in relation to cellular polarity. Part of the material of which it is composed is soluble in alcohol, becomes blackened after prolonged treatment with osmic acid, and, after appropriate preliminary fixation, shows a marked affinity for silver salts. In addition it may occasionally be stained with resorcin-fuchsin, iron hematoxylin, and other dyes, but the word "apparatus" is unfortunate because it carries with it the idea of a mechanism of a rather mechanical type. (Cowdry, 1924, p. 334)

Cowdry did report that the appearance of the Golgi apparatus was relatively constant in a particular cell type but varied across cell types so that "variations in its morphology are closely related to variations in cellular organization and function" (p. 334).

Variability in the appearance of the Golgi apparatus posed difficulties for cytologists. Kirkman and Severinghaus (1938a) reported that the following descriptions of the Golgi apparatus had been offered at different times: "a fibrous reticulum, network, ring, or cylinder, a very irregular fenestrated plate, a more or less incomplete hollow sphere, vesicle, or cup, a collection of small spheres, rodlets and platelets or discs, a series of anastomising canals, a group of vacuoles, and a differentiated region of homogeneous cytoplasm crossed by irregular interfaces" (p. 419).

I noted previously that Cajal correlated the appearance of the Golgi apparatus to cell secretion. Stronger evidence for its role in secretion stemmed from the work of Dimitry Nassonov (1923; 1924), who demonstrated the consistent

²¹ Dröscher (1998, p. 429), commented that Nusbaum "included 'Golgi' as a tribute to its discoverer, but eliminated the word 'reticulum' or 'net' because he and his collaborators at the University of Lemberg (Lwów) found that the disposition of the apparatus, especially in invertebrate cells, was not necessarily net-like, but predominantly in the form of single dictyosomes."

association of accumulating secretory products and the Golgi apparatus. He reported that granules for secretion first appeared in the meshes of the Golgi apparatus and proposed that after reaching a particular size, parts of the apparatus broke free and collected near the boundary of the cell. In a review paper in 1929, Robert Bowen defended the claim that the Golgi apparatus figured centrally in cell secretion, building up in the organelle and separating from it in different ways in different tissues.

In the first half of the twentieth century there was a long history of claims to the effect that the Golgi apparatus was an artifact of silver or osmium staining. Thomas Strangeways and R. G. Canti (1927) made such a case on the grounds that they were unable to find any evidence of the Golgi apparatus in unstained tissue-cultured cells either by direct or dark ground illumination. Many of these claims were followed by counterclaims. Richardson (1934), for example, claimed to find such evidence in cultured cells and contended that Strangeways and Canti were viewing cells in which the Golgi apparatus was fragmented. He as well as other investigators of the time also differentiated two parts to the Golgi apparatus – an outer part that absorbs osmium and silver and an inner portion that does not.

The strongest case for the claim that the Golgi apparatus was an artifact came from demonstrations that the preparation of cells for microscopy could result in a structure with the appearance of the Golgi apparatus. Maurice Parat (1928) proposed that the artifactual structure arose when fixatives caused certain cytoplasmic vacuoles to coalesce. John Baker (1944) further elaborated this view, holding that it actually was produced by deposition of metals (osmium or silver) on the periphery of the vacuoles. Walker and Allen (1927) used chemical models of gelatin, albumen, and lecithin to obtain evidence that the appearance of the Golgi apparatus resulted from the spreading of phospholipid materials on various interfaces produced during fixation. Gicklhorn (1932) claimed that laminated, doubly refractive myelin figures resembling the Golgi apparatus could be produced by treatment of isolated tonoplasts with methods developed by de Vries and suggested that the Golgi apparatus itself was produced by a similar release of myelin and the subsequent staining with silver or osmium.

On the other hand, the strongest evidence for the reality involved showing ways in which one could manipulate it experimentally. Beams and King (1934), applying Beams' centrifuge (see Chapter 4) to the uterine gland cells of guinea pig, caused the Golgi apparatus to appear to stream through the cytoplasm. They took this as indicating a fluid or semifluid character. Bourne (1942, p. 117) accepted this as compelling evidence "that the Golgi apparatus was a definite cell organ."

During this period suggestions of a possible role of the Golgi apparatus in secretion were further developed. Researchers found that it absorbed a variety of dyes into droplets, giving rise to the idea, articulated by Kirkman and Severinghaus, that the Golgi apparatus serves as a condensation membrane:

A great deal of work strongly suggests that the Golgi apparatus neither synthesizes secretory substances nor is transformed directly into them; but it acts as a condensation membrane for the concentration, into droplet or granules, of products elaborated elsewhere and diffused into the cytoplasm. These elaborated products may be lipoids, yolk, bile constituents, enzymes, hormones, or almost any other formed substance. (1938b, p. 85)

Others, however, argued that it was in fact the locus of synthesis – Bowen described it as “a great intracellular center of chemical synthesis or enzyme formation” (1924, p. 215). Bourne advocated the view that the surfaces of the Golgi apparatus were the key to their synthetic function.

Overall, by 1940 a consensus was developing that the Golgi apparatus was a true component of the cell, with most theorists proposing that it functioned in cell secretion.²² These claims, however, were still open to challenge. In Chapter 4 I note a pair of 1949 papers in which Albert Claude and George Palade argued that the Golgi apparatus was indeed an artifact of staining. Eventually they accepted the existence of this structure and, as discussed in Chapter 6, Palade made major contributions to determining its function.

The State of Cytology circa 1940

By 1940, cytology had advanced about as far as it could with the resources of the light microscope. The accounts of the mechanism of cell division remained the major success story in pursuit of mechanisms responsible for cell functioning. Although several organelles had been identified in the cytoplasm, each remained controversial. The evidence was strongest for the reality of the mitochondrion and the Golgi apparatus, but dissenters remained who

²² De Robertis, Nowinski, and Saez commented in their textbook, *General Cytology*, “Although the existence of a relationship between secretion and Golgi apparatus seems possible, the explanation of this relationship has not yet left the domain of hypothesis. If this is the present situation for the secretory cells, even more nebulous is the interpretation of the functional significance of the Golgi apparatus in the nonsecretory cells and particularly in the nerve cells, where it has such a considerable development. It has been thought that it may intervene in the secretion of fats, the elaboration of Nissle bodies, the metabolism of carbohydrates, and so on, but it is safer to affirm that up to the present time, there is no satisfactory theory to explain in a general form and for all cells the function of the Golgi apparatus” (1949, pp. 112–13; the same passage appears in the second edition, 1954, p. 147).

maintained that both were artifacts of the staining techniques used to see them. Even those who accepted the reality of the organelles disputed what function they played. A major reason both for the claims of artifact and for the disputes about function was that cytologists had limited resources to determine the function of organelles. The major strategy was to correlate frequency of the organelles' occurrence with activities the cell was performing. Many cytologists saw that the best prospect for moving beyond this state involved linkages with biochemistry. During the first half of the twentieth century, while cytologists were struggling, biochemists were making rapid advances.

2. BIOCHEMICAL CONTRIBUTIONS TO DISCOVERING CELL MECHANISMS UP TO 1940

Biochemistry established itself in the early decades of the twentieth century in what was then underdeveloped territory between physiology and chemistry (Kohler, 1973; Kohler, 1982). It drew upon considerable research on chemical processes in living organisms in the nineteenth century, but did not develop its own methods and conceptual framework until the beginning of the new century. I will begin by briefly reviewing some of the foundations that were laid in the nineteenth century, discuss what distinguished biochemistry as it emerged in the twentieth century, and then describe in greater detail research on glycolysis (fermentation) and aerobic respiration in the period 1900 to 1940.

Foundations for Biochemistry in the Nineteenth Century

The first attempts to build bridges between chemistry and activities of living organism preceded the chemical revolution at the end of the eighteenth century, but with the emergence of a new chemistry outlined by Antoine Lavoisier these efforts acquired a new foundation.²³ Lavoisier himself contributed significantly to charting the path that subsequent investigators would follow. The new systemization of the basic elements led to analyzing organic substances in terms of these elements. Lavoisier (1781) determined that carbon, hydrogen, and oxygen are constituents of all living organic substances. Claude Louis Berthollet (1780) identified nitrogen as another frequently occurring component. With these foundations, investigators began trying to characterize physiological processes in terms of changes in elemental composition.²⁴ For

²³ For more detailed analysis, see Holmes (1963).

²⁴ Since different organic substances were composed from the same elements, many chemists concluded that they differed from one another only in terms of their relative proportions. Thus,

example, Lavoisier (1789) himself characterized fermentation as involving the oxygenation of carbon in sugar to produce carbon dioxide at the expense of the deoxygenation of the remainder, yielding alcohol. Shortly thereafter Louis Jacques Thénard (1803) and subsequently Joseph Louis Gay-Lussac (1810) worked out the general formula for fermentation, represented in modern notation as



At the time yeast was not regarded as an organism, so fermentation, although involving organic compounds, was regarded as a strictly chemical process. In further research in collaboration with Pierre Simon LaPlace, Lavoisier measured the heat generated as animals respired and compared that with the heat of combustion of coal that generated the same amount of carbon dioxide. They concluded, “Respiration is thus a very slow combustion phenomenon, very similar to that of coal” (Lavoisier & LaPlace, 1780, p. 331 in 1886 reprint).

In the succeeding decades, a host of chemists turned their attention to the phenomena exhibited by living systems. One of the challenges they confronted is that chemical reactions occur in living organisms that do not freely occur outside of them. In some cases chemists identified and isolated substances that promoted reactions without being consumed in them. Gottlieb Sigismund Kirchhoff (1816) had shown that germinating grains of malt facilitated the conversion of starch to sugar. Payen and Persoz (1833) later extracted the chemical component that facilitated the process and named it *diastase*. Jöns Jacob Berzelius (1836) introduced the term *catalysis* for the process and, appealing to inorganic examples, proposed to apply the idea to fermentation.

This property was not an isolated, exceptional behaviour but proved to be a more general one, exhibited by substances to varying extents.... We have found, for instance, that the conversion of sugar to carbon dioxide and alcohol, which occurs in fermentation through the influence of an insoluble substance known by the name of ferment... could not be explained by a chemical reaction between sugar and ferment resembling double decomposition. However, when compared with phenomena known in inorganic Nature, the preceding phenomenon most

Antoine François de Fourcroy concluded that substances such as oils, acids, mucilages, and fibres “differ from each other only in the number and the proportions in which the primary substances are combined in them” (Fourcroy, 1789, translation by Holmes, 1963, p. 57). One consequence of this research was the determination that nitrogen was present in far higher concentrations in animal tissue than plant tissue, inspiring the hypothesis that plant tissue had to be animalized by increasing the concentration of nitrogen to generate animal tissue.

closely resembles the decomposition of hydrogen peroxide under the influence of platinum, silver or fibrin; it was hence very natural to imagine an analogous activity in the case of the ferment. (passage translated in Friedmann, 1997, p. 74)

In the same year, Schwann (1836) isolated pepsin, a catalyst in gastric juice that breaks down proteins. Chemists also sought to synthesize substances produced by living organisms. Friedrich Wöhler (1828), for example, synthesized urea from ammonia and cyanic acid. Wöhler's enthusiasm was evident in a letter he wrote to Berzelius: "I can no longer, as it were, hold back my chemical urine; and I have to let out that I can make urea without needing a kidney, whether of man or dog" (quoted in Friedmann, 1997, p. 68).

With the tools of elementary analysis and the concept of catalysis and the example of Wöhler's success in synthesizing urea, some investigators saw the time as ripe to formulate a detailed chemical account of all processes occurring in living organisms. In many respects, the most ambitious of these attempts came from Justus Liebig. After studying in Paris with Thénard and Gay-Lussac, among others, Liebig became a professor at the University of Giessen in 1824 and established one of the most influential laboratories for the study of chemistry. One of his first accomplishments was to perfect an instrument for the elemental analysis of organic substances (Liebig, 1831). A further inspiration for Liebig's thinking was William Prout's (1827) classification of the nutrients required by animals into three classes: saccharine (carbohydrates), oleaginous (fats), and albuminous (proteins). Prout had noted that there were only minor differences in chemical composition between the nutrients animals took in from plants and the compounds that comprised the fluids and solids of their bodies. Liebig drew upon this observation to formulate a central part of the synthetic and highly speculative account of the chemical processes of animals in his *Animal Chemistry* (1842). He proposed that animals incorporated nutrients into their bodies and, as needed, broke them down to their constituents. Because animal tissues were primarily made of protein, he hypothesized they reconstituted them by simply incorporating protein from their diet. When muscle work was required, these proteins were broken down, with waste products excreted. In contrast, he thought animals burned carbohydrates and fats to generate heat. When insufficient oxygen was available for burning, animals converted them to fat and stored them. With these key ideas, Liebig articulated a general scheme, complete with detailed formulae,²⁵ that described the chemical operations occurring in animals.

²⁵ These detailed formulae often aroused skepticism. Even though Liebig dedicated *Animal Chemistry* to Jacob Berzelius, Berzelius derided it as "physiological chemistry . . . created at the writing table" (quoted in Fruton, 1972, p. 97).

Liebig's approach to physiological chemistry promised to reveal the chemical events in animals without requiring direct empirical investigation of internal operations. This was only plausible because Liebig had assumed that all processes in animals were catabolic – breaking down complex substances into simple ones but creating nothing new. However, when Claude Bernard set out to determine where the oxidation of carbohydrates occurred in animals, he discovered that in fact glycogen was being synthesized. This surprising discovery revealed the oversimplification in Liebig's scheme (Bernard, 1848). This was a major inspiration for Bernard's very different conception of metabolic processes. As I discussed in Chapter 2, Bernard (1878a, p. 113) proposed that each organ in an organism performs one of the operations necessary to maintain the "constancy of the internal environment."

When he coined the concept of a catalyst, Berzelius had assumed that it would be a relatively straightforward project to generate a chemical account of fermentation. The discovery by Schwann, as well as Charles Cagniard-Latour (1838) and Friedrich Traugott Kützing (1837), that yeast were living suddenly made fermentation a more challenging case for those seeking to provide chemical accounts of physiological processes.²⁶ Louis Pasteur followed

²⁶ Liebig, Berzelius, and Wöhler perceived the threat to the program of giving chemical accounts of physiological processes, and their response was extremely harsh. Wöhler published excerpts of a paper by Turpin (1838) following up on Cagniard-Latour's research in *Annalen der Pharmacie*, which he and Liebig edited. Following the excerpts he published a heavy-handed satire (officially anonymous, but clearly the work of Wöhler, perhaps with the collaboration of Liebig) titled "The demystified secret of alcoholic fermentation," which purported to describe detailed observations with a special microscope: "Incredible numbers of small spheres are seen which are the eggs of animals. When placed in sugar solution, they swell, burst, and animals develop from them which multiply with inconceivable speed. The shape of these animals is different from any of the hitherto described 600 species. They have the shape of a Beindorf distilling flask (without the cooling device). The tube of the bulb is some sort of a suction trunk which is covered inside with fine long bristles. Teeth and eyes are not observed. Incidentally, one can clearly distinguish a stomach, intestinal tract, the anus (as a pink point), and the organs of urine excretion. From the moment of emergence from the egg, one can see how the animals swallow the sugar of the medium and how it gets into the stomach. It is digested immediately, and this process is recognized with certainty from the elimination of excrements. In short, these infusoria eat sugar, eliminate alcohol from the intestinal tract, and CO₂ from the urinary organs. The urinary bladder in its filled state has the shape of a champagne bottle, in the empty state it is a small bud. After some practice, one observes that inside a gas bubble is formed, which increases its volume up to tenfold; by some screw-like torsion, which the animal controls by means of circular muscles around the body, the emptying of the bladder is accomplished . . . From the anus of the animal one can see the incessant emergence of a fluid that is lighter than the liquid medium, and from their enormously large genitals a stream of CO₂ is squirted at very short intervals . . . If the quantity of water is insufficient, i.e. the concentration of sugar too high, fermentation does not take place in the viscous liquid. This is because the little organisms cannot change their place in

Schwann and the others in relating fermentation to the activity of living yeast cells: “Fermentation is correlated to the vital processes of yeast” (Pasteur, 1860, p. 323). For him and for many, this pointed to the futility of trying to provide a purely chemical account of fermentation. But despite Pasteur’s influence, numerous researchers (e.g., Pierre Eugène Marcellin Berthelot, Moritz Traube, Felix Hoppe-Seyler) contended that it was a chemical constituent of yeast (as pepsin is a chemical substance in the stomach) that catalyzed the reaction. Wilhelm Kühne (1877a; 1877b) introduced the term *enzyme* (from “in yeast” in Greek), which eventually came to be the term in general use for such agents.²⁷ Until Buchner’s breakthrough in 1897 (discussed below), however, there was no compelling evidence that enzymes – that is, chemical agents – were responsible for fermentation.²⁸

During this period in which convincing empirical results supporting a chemical approach to fermentation were not forthcoming, chemically minded researchers focused on the mechanisms of animal respiration. Lavoisier’s research had opened a prolonged conflict about whether respiration occurred in animals’ lungs (Lavoisier), blood (Liebig, Bernard), or tissues (Pflüger). (For further discussion of this conflict, see Bechtel & Richardson, 1993, Chapter 3.) Eduard Pflüger (1872; 1875) ultimately provided compelling evidence that respiration occurred in the tissues. Settling this question opened the search for the mechanism within tissues that made respiration possible. Pflüger proposed that the protoplasm that comprised the cells of tissues had a complex physical structure that resulted from the polymerization of protein. He further proposed that this structure stored energy that could be released in “explosions” in the cell: “The life process is the intramolecular heat of highly unstable albuminoid molecules of the cell substance, which dissociate largely with the formation of carbonic acid, water, and amide-like substances, and

the viscous liquid: they die from indigestion caused by lack of exercise” (Wöhler, 1839; passage translated in Schlenk, 1997, p. 47).

²⁷ For Kühne the term marked a contrast between chemical agents and *organized ferments*, living organisms that produced chemical changes. The eventual use of the term *enzyme* for chemical agents thus reversed Kühne’s intention in coining the term.

²⁸ After Bernard’s death in 1878 a manuscript was found in his country house proposing that alcoholic fermentation resulted from a soluble ferment found in ripe or rotting fruit (Bernard, 1878b). It was not clear whether Bernard was reporting actual results. This manuscript provoked Pasteur to pursue an extensive attempt to isolate such an enzyme and an extended exchange between Bertholet and Pasteur (For a review and discussion, see Friedmann, 1997; Kohler, 1971). About the same time, Marie Mikhailovna Manassein (1872) reported in the same journal in which Buchner was to publish that she had succeeded in producing fermentation in a cell-free extract. Her brief report, however, did not elicit much response.

which continually regenerate themselves and grow through polymerization” (Pflüger, 1875, p. 343, as quoted in Fruton, 1972, p. 284).

As Pflüger’s proposal exemplifies, theorizing about metabolic mechanisms in the later part of the nineteenth century was highly speculative. In large part, this was because proteins are extremely complex and it was not possible at the time to establish that they were even molecular in character. Whereas organic chemists focused on relatively simple molecules in order to make progress in articulating the structure of organic compounds, researchers interested in the chemistry underlying physiological functions emphasized the likely importance of complex organization. One avenue toward complexity was offered by colloid chemistry, which emphasized molecules organized in arrays on surfaces. It became increasingly influential after Thomas Graham (1861) proposed that protoplasm was composed of colloidal arrays rather than less structured molecules. Investigators such as Wolfgang Ostwald (1909) emphasized the multiple phases of colloidal dispersion and the energies associated with surfaces. Some historians of biochemistry, such as Marcel Florkin (1972, pp. 279–83), have bemoaned this as the “dark age of biocolloidology.” Florkin portrayed the influence of colloid chemistry on physiology as hindering the advance of a theoretically sound biochemistry and so drew a sharp contrast between colloidal chemistry and biochemistry. Yet, even as major a contributor to biochemistry as Otto Warburg began his career emphasizing the importance of colloidal surfaces for cell respiration (Warburg, 1911; Warburg, 1913b).²⁹

The Emergence of Biochemistry in the Twentieth Century

The pioneering efforts to provide chemical explanations of vital processes in the nineteenth century suddenly developed into a robust research endeavor in the early twentieth century. At this time, the term *biochemistry* began to supplant older labels such as *physiological chemistry*. In the first decade of the twentieth century, new journals developed with *biochemistry* in their titles³⁰ and new professional societies were established.³¹ In 1909 Carl Oppenheimer

²⁹ Warburg was generally dismissive of the emphasis on enzymes in mainstream biochemistry and pointedly referred to them using an older term, *ferment*. He identified one such important substance himself, which he called *Atmungsferment* (now known as cytochrome oxidase).

³⁰ In 1902 *Biochemisches Centralblatt, vollständiges Sammelorgan für die Grenzgebiet der Medizin und Chemie*; in 1903 *Beiträge zur chemischen Physiologie und Pathologie, Zeitschrift für die gesamte Biochemie*; in 1905 *Journal of Biological Chemistry*; in 1906 both the *Biochemical Journal* and *Biochemische Zeitschrift*.

³¹ In 1905 the American Chemical Society initiated a section of biological chemistry and in 1911 in England the Biochemical Club – later to become the Biochemical Society – was founded.

published his *Handbuch der Biochemie* in which he offered the following characterization:

Biochemistry is the science that deals first with the constituents of living tissues, and their determination, properties, and reactions; but which also strives to draw from the chemical changes that proceed during life processes, conclusions regarding the scope of the life-process itself. (Oppenheimer, 1909, pp. v–vi)

Historians have proposed two major factors as differentiating the newly emerging biochemistry from the physiological chemistry of the nineteenth century that I have been describing. Robert Kohler (1973) emphasized the strategy of identifying different enzymes responsible for each reaction.³² As we have just seen, investigators had already identified a host of catalysts responsible for physiological operations before Kühne coined the term, and by the 1890s physiologists generally accepted that digestion in animals was the work of enzymes. Emil Fischer's (1894) research and his lock-and-key model of enzyme action had revealed the highly specific nature of enzyme action. All of the reactions for which enzymes had been discovered to that point, however, were simple hydrolytic ones (that is, they consumed water in reactions that split apart organic substances). What Kohler emphasized is that until the 1890s no catalysts had been identified as involved in the more complex reactions of cells such as respiration or fermentation and none had been demonstrated to operate within cells (all enzymes known at that time were secreted by cells and operated outside).

Kohler presented Gabriel Bertrand (1895) as having taken the first step beyond hydrolytic enzymes when he determined that a catalyst he identified in the Chinese art of making black lacquer finish, laccase, caused the uptake of oxygen and the production of carbon dioxide. Bertrand compared the process to artificial respiration.³³ The key step in demonstrating that enzymes could operate within cells came with Eduard Buchner's (1897) report (discussed below) that in a cell-free extract made by grinding yeast cells with sand, fermentation still occurred. Appealing to enzymes rather than protoplasm to explain processes in living organisms, according to Kohler, marked the major change from physiological chemistry to biochemistry: “the very language of

³² Kohler cites Franz Hofmeister (1901) as giving voice to the view that all reactions could be explained by enzymes: “we may be almost certain that sooner or later a particular specific ferment will be discovered for every vital reaction” (p. 14).

³³ Kohler identified a second major expansion of the notion of enzymes, occurring with Arthur Croft Hill's (1898) discovery that maltase, in appropriate circumstances, synthesized maltose from glucose rather than cleaving maltose to form glucose.

biochemistry changed when enzymes replaced protoplasm as the seat of vital chemistry” (1973, p. 193).

Frederic Holmes (1986; 1992) emphasized a second factor that differentiates biochemistry from its predecessors, the conception of intermediary metabolism as involving sequences of specific chemical reactions. This established the goal of discovering each intermediate reaction.³⁴ This had been a goal of many early chemists, but one that was difficult to realize when the units for chemical analysis remained the elements of which organic substances were composed. (Recall Liebig trying to describe the chemical operations in animals by balancing chemical formulae.) An important contribution of organic chemistry in the last part of the nineteenth century was to identify higher-level structural components of organic molecules whose addition, removal, or transfer characterized the basic reactions biochemistry would examine.

By 1900, organic compounds relevant to metabolic processes could be characterized structurally. Characteristic groups such as hydroxyl, carboxyl, and amino groups were linked at specific sites to the carbon backbones of organic molecules. The general classes of reaction mechanisms in which each reactive group participated, and some of the influences of their relative placements on the carbon skeleton upon their reactivities, were known. This information provided strong foundations for interpreting the chemical changes linking any compounds that could be shown to take part in metabolic processes. (Holmes, 1992, p. 56)

Biochemists also needed techniques for demonstrating intermediate reactions involving such structural units. Because the intermediate products generally did not accumulate but were immediately metabolized, it was a challenge to produce empirical evidence of the intermediate steps. Holmes identified Franz Knoop as taking a major step in 1904 when he fed animals compounds similar to normal foods but ones they could not fully metabolize. Knoop found that after feeding dogs specific aromatic derivatives of fatty acids, their urine would contain an aromatic acid that was shorter by an even number of carbon compounds (unless the fatty acid chain contained only one carbon, in which case it was not broken down). He proposed a mechanism in which fatty acids

³⁴ Noting that Kohler had cited Hofmeister (1901) for emphasizing the importance of enzymes, Holmes (1992, p. 58) quoted additional passages in which Hofmeister stated that chemical reactions in cells “must take place in specific intermediate steps” which can be expressed “through chains of physical and chemical formulas.”

are decomposed by the removal of two carbon atoms at a time, a process he named β -oxidation (Knoop, 1904).³⁵

In the first four decades of the twentieth century biochemists made great strides in developing sketches of the mechanisms underlying many metabolic processes. In particular, by 1940 they had developed detailed accounts of the biochemical mechanisms involved in fermentation (glycolysis) and aerobic cellular respiration. The research on fermentation was a singular success story for biochemistry, largely because the reactions occur freely in the cytoplasm and do not depend on cellular organization. With respect to aerobic respiration, biochemists were successful in identifying the major pathways, but their inability to carry out the full process in preparations that did not include membranes suggested a linkage with cell structure that they lacked the tools to investigate. They also faced problems in explaining the generation of ATP from the component operations of respiration and it turned out this step depended critically on the linkage to cell structure.

Alcoholic and Lactic Acid Fermentation (1895–1940)

As noted previously, the orthodox view that fermentation required living yeast was finally undercut by Eduard Buchner in 1897. Buchner had added sugar to what he termed *press juice* (later called *yeast juice* or *cell-free extract*), made by adding water to ground yeast, and then filtering it under pressure. He made his discovery while collaborating with his brother, Hans Buchner, in grinding bacterial cells with the goal of isolating proteins that might be serving as the active bacterial agents. The endeavor was initially obstructed by the inability to remove remnants of cell bodies from the preparation, but in 1896 Hans Buchner's collaborator, Martin Hahn, developed a technique for filtering the cell structure debris from ground yeast. The Buchners added sugar to the resulting juice, thinking it would serve as a preservative, but the resulting formation of carbon dioxide made Eduard Buchner realize that they had achieved something of far more importance: cell-free fermentation.

Initially Buchner took fermentation to be a single chemical reaction, and coined the name *zymase* for the responsible enzyme. In further research, though, Buchner found evidence that “lactic acid plays an important role in

³⁵ Holmes noted that Knoop in this publication set for himself the “ultimate goal of a complete knowledge of the cleavage and oxidative processes of the building materials and foodstuffs of the animal body” (Knoop, 1904, p. 3, translated by Holmes, 1992, p. 59).

the cleavage of sugar and probably appears as an intermediate in alcoholic fermentation” (Buchner & Meisenheimer, 1904, pp. 420–1). This led him to propose that alcoholic fermentation was a two-step process, with zymase catalyzing the reaction from glucose to lactic acid and another enzyme, lactacidase, catalyzing the reaction from lactic acid to alcohol.

The proposal that lactic acid figures in alcoholic fermentation was particularly interesting because it was the product of a different process, that associated with the souring of milk, which Pasteur (1858; 1857) had also investigated and interpreted as analogous to alcoholic fermentation. As well, Emil du Bois-Reymond (1859) had discovered the presence of lactic acid after muscle contraction or after death of an animal. The linkage between lactic fermentation and muscle action was a continuing focus of research in succeeding decades. The linkage was not well-established, though, until Fletcher and Hopkins (1907) showed that the formation of lactic acid during anaerobic muscle contraction was followed by its removal in the aerobic phase.

Lactic acid, however, was soon discredited as an intermediary in alcoholic fermentation on the grounds that adding it to yeast failed to generate alcohol (Slator, 1906). The nature of the relation between alcoholic fermentation and lactic fermentation was not revealed until later. Research seeking intermediaries of alcoholic fermentation turned rather to several three-carbon sugars which had been identified by organic chemists who had attacked glucose with alkalis – methylglyoxal, glyceraldehyde, and dihydroxyacetone. As well, Otto Neubauer, while investigating amino acid metabolism, identified pyruvic acid as an intermediary in that process and proposed it also figured in alcoholic fermentation (Neubauer & Fromherz, 1911). Other researchers quickly corroborated this finding and determined that it was decarboxylated to yield acetaldehyde.

The emerging challenge for biochemists was both to determine which of the possible intermediates figured in the fermentation pathway and to develop a model of the pathway that related those that did occur using only known chemical operations such as oxidations, reductions, and decarboxylations. Carl Neuberg developed a comprehensive model of a sequence of reactions for generating alcohol from glucose (Neuberg & Kerb, 1914). As shown in Figure 3.6, he proposed that glucose was scissioned into two molecules each of methylglyoxal and water. The methylglyoxal then reacted with acetaldehyde (produced in a previous iteration of the process) and water, generating both pyruvic acid and alcohol. The pyruvic acid was then decarboxylated to acetaldehyde. (Neuberg also proposed an alternative route by which methylglyoxal would generate glycerol and pyruvic acid, which could then be decarboxylated to provide the initial quantity of acetaldehyde.) Neuberg’s model

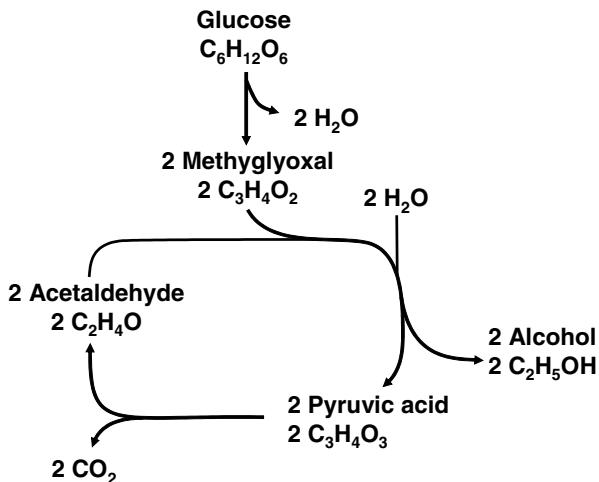


Figure 3.6. A representation of Neuberg and Kerb's proposed pathway of fermentation that brings out how it constituted a cycle.

was the focus of extensive research during the next fifteen years, much of it directed to showing that the proposed intermediates, especially methylglyoxal, really figured in the pathway. Because intermediates typically would not accumulate, indirect evidence was required. One critical issue was whether the intermediate would react to form alcohol at least as rapidly as glucose. Methylglyoxal failed this test, leading Neuberg to propose that it was an isomer of methylglyoxal that was the true intermediate.

Neuberg's proposal also failed to explain an additional finding concerning fermentation in cell-free extracts. Fermentation in such extracts typically slowed dramatically in a short time. Arthur Harden (1903) established that adding blood serum would produce an 80% increase in fermentation. Together with William Young, Harden further demonstrated that adding phosphate would also stimulate the reaction, which would then slow down again when the phosphate was exhausted (Harden & Young, 1908). They also established that the phosphate appeared to be taken up into a hexosediphosphate ester that itself could not be further metabolized but, as illustrated in Figure 3.7, would slowly decompose through hydrolysis. Neuberg dismissed this evidence, though, on the grounds that hexosediphosphate would not ferment in living cells (Neuberg & Kobel, 1925), thereby happily invoking the same argument strategy whose conclusion he resisted in the case of methylglyoxal. Harden and Young also demonstrated the need for addition of a "dialyzable substance which is not destroyed by heat" to maintain cell-free fermentation (1906, p. 410). Because heat destroyed the enzyme itself, this was obviously an additional substance;

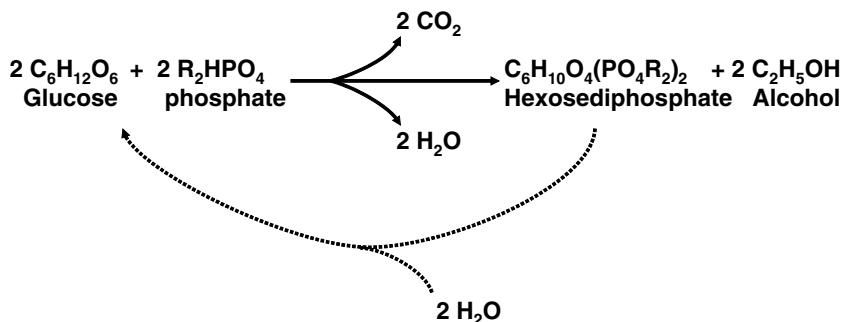


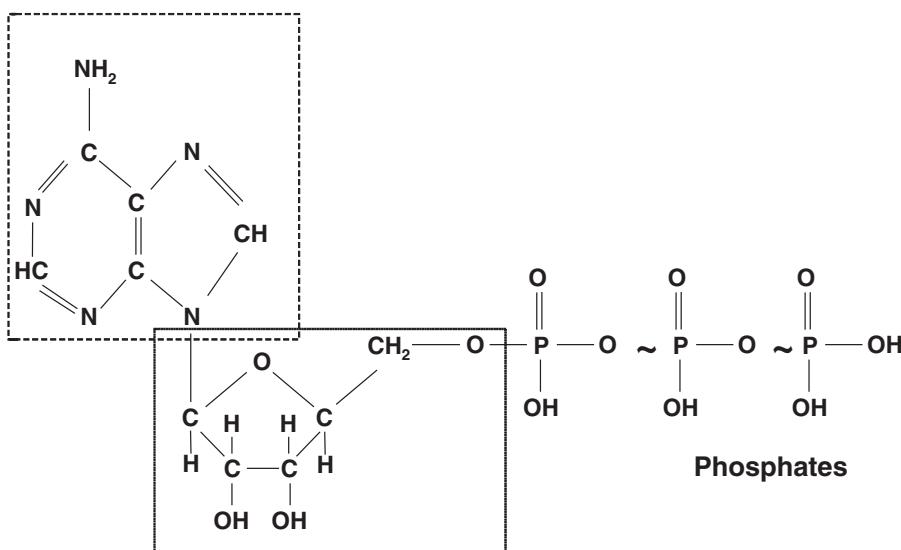
Figure 3.7. Harden and Young's conceptualization of how phosphates might figure in fermentation. Two molecules of glucose would react with two phosphate molecules to create one hexosediphosphate ester and two molecules of alcohol, with the hexosediphosphate slowly breaking back down to glucose and two phosphates, which would then be able to participate in reactions with another molecule of glucose.

they called it a “coferment.” Its significance became apparent only in the 1930s (see below).

While researchers investigating alcoholic fermentation were puzzling about the roles of methylglyoxal and phosphates, Gustav Embden was investigating lactic acid fermentation in press juice from muscle. Because adding glucose failed to increase the yield of lactic acid, he proposed that the lactic acid was derived not directly from glucose but from an unknown precursor he designated *lactacidogen* (Embden, Kalberlah, & Engel, 1912). He soon found that adding hexosediphosphate resulted in a large increase in lactic acid and suggested that it was related to *lactacidogen* (Embden & Laquer, 1914; Embden & Laquer, 1921). This work by Embden, together with investigations by Otto Meyerhof (1918) demonstrating that very similar coferments were required in alcoholic fermentation and lactic acid fermentation, pointed strongly to a close connection between the two processes. Meyerhof coined the term *glycolysis* to cover both reaction pathways.

A key to the mysteries surrounding alcoholic fermentation and muscle fermentation was provided by the discovery of two new phosphorus compounds in the late 1920s. The first occurred when Philip Eggleton and Marion Grace Palmer Eggleton (1927) isolated a substance known initially as *phosphagen* and later as *phosphocreatine*. What distinguished this substance was that it rapidly hydrolyzed (i.e., broke down to creatine and phosphate with the consumption of a molecule of water), and released large quantities of energy as it did so. This indicated that it might provide the immediate source of energy for muscle contraction. Eimar Lundsgaard confirmed this hypothesis by showing that in iodoacetate-poisoned rabbits, muscle activity prior

Adenine



Ribose

Figure 3.8. Chemical structure of adenosine triphosphate (ATP). When the high-energy bonds (\sim) are broken, a phosphate group is released as well as 14 kcal, about twice as much energy as released from an ordinary covalent bond (—).

to the onset of rigor mortis resulted not in accumulation of lactic acid but rather in the breakdown of phosphocreatine. Around the same time, Cyrus Fiske and Yellapragrada Subbarow (1929) and Karl Lohmann (1929) discovered another phosphorus compound initially called *adenylphyrophosphate* and now known as *adenosine-triphosphate* (ATP; its chemical structure is shown in Figure 3.8). It too would undergo rapid hydrolysis (losing one phosphate group at a time) with release of considerable energy. Together with Meyerhof, Lohmann demonstrated that ATP was one of the coenzymes contributing to Harden and Young's undifferentiated coferment of yeast juice (Meyerhof, Lohmann, & Meyer, 1931). As well, Vladimir Englehardt (1932) established a linkage between ATP formation and aerobic respiration.³⁶

These discoveries of phosphocreatine and ATP pointed to a crucial role for phosphorylated compounds among the products of muscle glycolysis and

³⁶ In studies on pigeon erythrocytes, Englehardt found that when he supplied cyanide, a respiratory inhibitor, ATP rapidly broke down and inorganic phosphate built up, but that ATP was resynthesized after the cells were washed. In non-respiring rabbit erythrocytes, on the other hand, the buildup occurred when he used fluoride to inhibit glycolysis.

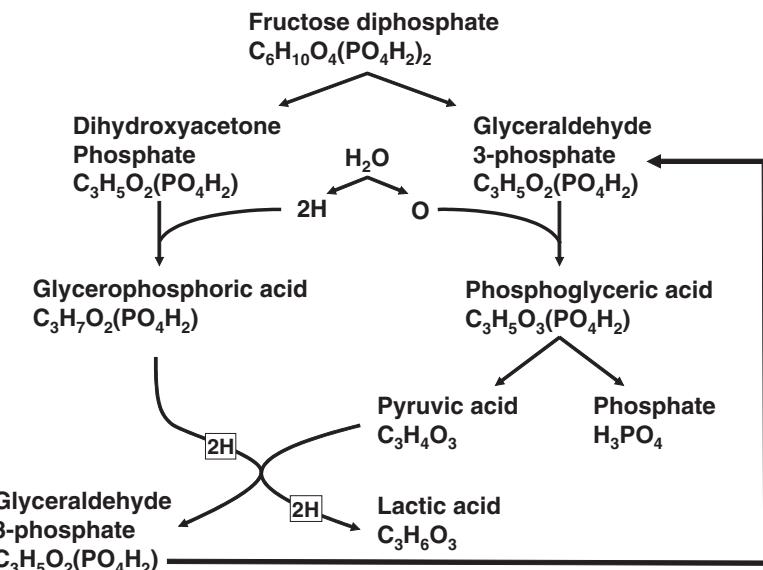


Figure 3.9. A representation of how Embden conceptualized the process of glycolysis as involving phosphorylated compounds throughout. Fructose diphosphate is first scissioned into two triosephosphates, one of which, glyceraldehyde-3-phosphate, is oxidized while the other, dihydroxyacetone phosphate, is reduced. The oxidation product, phosphoglyceric acid, then surrenders its phosphate to produce pyruvic acid. For muscle glycolysis, the product of the reduction, glycerophosphoric acid, is then oxidized at the expense of reducing pyruvic acid to lactic acid, yielding more glyceraldehyde-3-phosphate, which can reenter the reaction at an earlier step.

suggested that Harden and Young's discovery of the need for phosphates in order to maintain fermentation in a cell-free environment might reflect a more fundamental feature of glycolysis. In a paper published posthumously, Embden (Emden, Deuticke, & Kraft, 1933) produced a scheme for muscle glycolysis, illustrated in Figure 3.9, in which phosphorylated forms of several three-carbon sugars served as intermediaries. The key step in the pathway was the oxidation of one of the triosephosphate molecules to 3-phosphoglyceric acid at the expense of the reduction of the other to glycerophosphoric acid. Embden did not discuss the significance of the product of oxidation being phosphorylated, but in the next year Jacob Parnas proposed that the phosphate was not simply liberated, but transferred to phosphocreatine (Parnas, Ostern, & Mann, 1934).³⁷ (It was later determined that in fact it is transferred to ADP.)

³⁷ Parnas commented, "...the resynthesis of phosphocreatine and adenosine triphosphate is not linked to glycolysis as a whole, but to definite partial processes: and this leads further to the

The accomplishments of Embden and Parnas radically reshaped thinking about alcoholic fermentation and muscle glycolysis, making central the linkages between oxidation and energy by construing the process in terms of phosphorylated compounds and proposing the transfer of phosphate to ADP to form ATP. Several years later Fritz Lipmann (1941) would introduce the symbol $\sim P$ to designate what he memorably called the “energy-rich phosphate bonds” in ATP (p. 101). Already in the 1930s, though, the connections linking oxidation, phosphates, and energy were becoming apparent. In this respect, an important step was Dorothy Needham’s proposal of a second esterification of phosphate in fermentation. She noted that even when fluoride poisons blocked the step from phosphoglyceric acid to phosphopyruvic acid (which by then had been identified as an intermediate before the production of pyruvic acid), free phosphate continued to be taken up into an ester. She also observed that in normal fermentation more phosphocreatine was formed per molecule of lactic acid than the single transfer of phosphate from phosphopyruvic acid to ATP could explain. Accordingly, she proposed a second synthesis of ATP (Needham, 1937), and Negelein and Brömel (1939) demonstrated that this involved first the formation of diphosphoglyceric acid (3-phosphoglyceroyl phosphate) as the immediate oxidation product of glyceraldehyde 3-phosphate.

One last change resulted in the conception of the pathway as still accepted today. In 1934 Warburg and Christian found another constituent beyond ATP in a coferment preparation made from red blood cells. In 1935 they identified it chemically as containing nicotinic acid amide (Warburg & Christian, 1935). Soon thereafter they characterized it functionally as a “hydrogen-transporting co-ferment” (Warburg, Christian, & Griese, 1935) and proposed the name *triphosphopyridine nucleotide (TPN)* (Warburg & Christian, 1936). The main purpose of this 1936 article, though, was to announce their isolation of a similar coferment with just two, rather than three, molecules of phosphoric acid per molecule of nicotinamide. They proposed the name *diphosphopyridine nucleotide (DPN)*; this key substance has also been known as *coenzyme I*, *nicotinamide adenine dinucleotide (NAD)*, and, in its oxidized/reduced states, $NAD^+/NADH$.³⁸ Meyerhof then established that it was actually NAD^+ , not

conclusion that this resynthesis does not involve a relationship that might be termed ‘energetic coupling,’ but more probably involves a transfer of phosphate residues from molecule to molecule” (Parnas et al., 1934, p.68).

³⁸ More specifically, the two coenzymes (“co-ferments”) discovered by Warburg and Christian are pyridine nucleotides that have in common two phosphate-containing nucleotides (nicotinic amide mononucleotide and adenine flavin dinucleotide); however, DPN lacks an additional

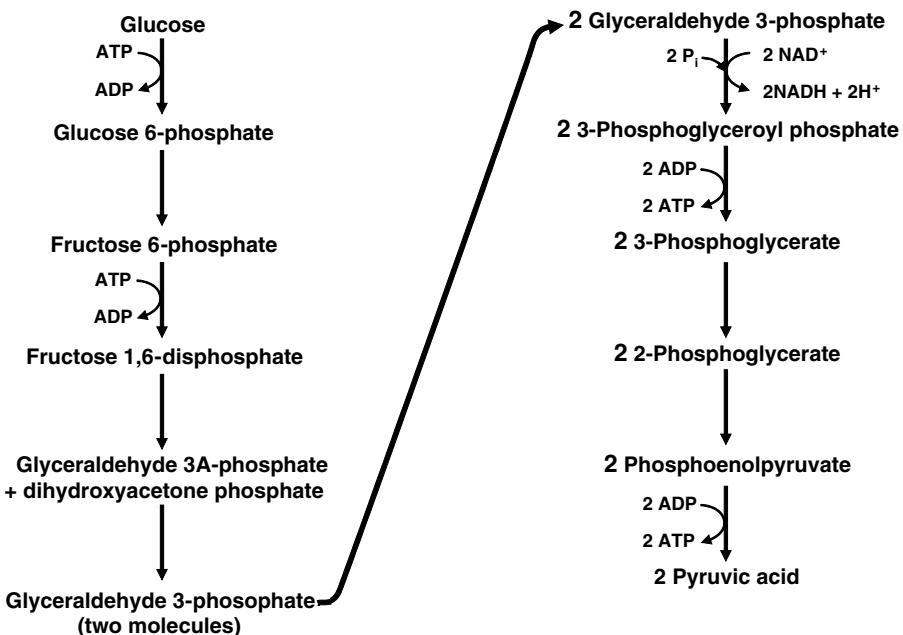


Figure 3.10. The complete Embden-Meyerhof pathway of glycolysis. The reactions in the left-hand column are often characterized as *preliminary*, transferring energy from ATP to products of glucose and splitting the molecule into two molecules of glyceraldehyde-3-phosphate. The reactions in the left-hand column involve the oxidation step and the subsequent transfer of energy to ATP.

dihydroxyacetone phosphate, that was reduced (took up two hydrogen atoms, making $\text{NADH} + \text{H}^+$) in conjunction with the oxidation of glyceraldehyde 3-phosphate (Meyerhof, Ohlmeyer, & Möhle, 1938).³⁹ NADH thus took over the role of glycerophosphoric acid in Embden's scheme, serving as the hydrogen donor in the reduction of pyruvic acid to lactic acid in muscle glycolysis and to alcohol in alcoholic fermentation.

With this final contribution of Meyerhof, what is known as the Embden-Meyerhof pathway (sometimes the Embden-Parnas-Meyerhof pathway) achieved its mature form as shown in Figure 3.10. The reactions from glucose

phosphate radical that is found in *triphosphopyridine nucleotide (TPN)*, also known as *coenzyme II*. The modern terms *nicotinamide adenine dinucleotide (NAD)* and *nicotinamide adenine dinucleotide phosphate (NADP)* were adopted as part of an international agreement in the 1960s (see Afzelius, 1966, p. 12, fn).

³⁹ Only one hydrogen atom is actually added to NAD^+ ; the second dissociates into H^+ and an electron, with the electron being incorporated along with the hydrogen atom into NADH .

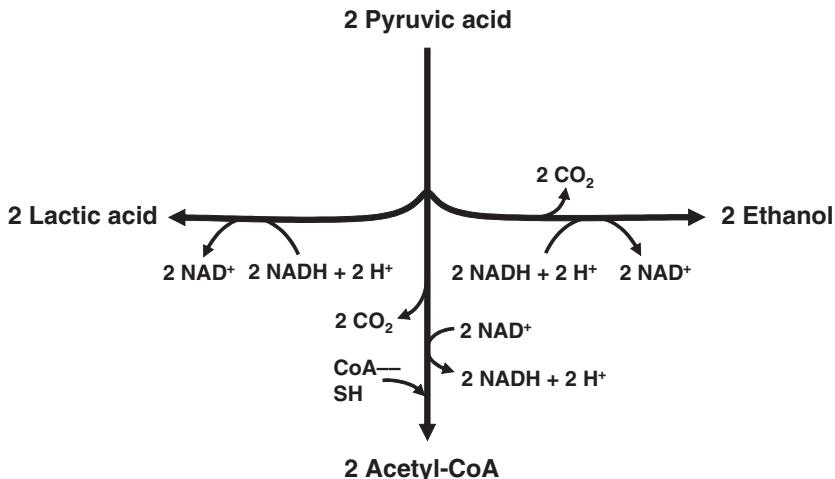


Figure 3.11. Three alternative pathways from pyruvic acid. In anaerobic environments, pyruvic acid is reduced to lactic acid (shown on the left) or to alcohol with the liberation of carbon dioxide (shown on the right). In aerobic conditions, it is decarboxylated, further oxidized, and combined with coenzyme A (CoA-SH) to produce acetyl-CoA, which then enters the citric acid cycle.

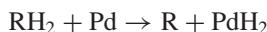
to pyruvic acid are common to both alcoholic and lactic acid fermentation and are generally referred to, following Meyerhof's proposal, as *glycolysis*. The pathways diverge in the steps after pyruvic acid (Figure 3.11). Although I have not emphasized the point, researchers along the way named enzymes and typically provided indirect evidence for their operation, so that glycolysis came to be viewed as a complex sequential pathway in which each operation in turn was catalyzed by a specific enzyme and resulted in a product that served as substrate of the next operation. As such, glycolysis became the exemplar of how to understand physiological processes in biochemical terms.

Aerobic Cellular Respiration (1910–1940)

In the nineteenth century, researchers made a sharp distinction between fermentation, which occurred under anaerobic conditions, and aerobic cellular respiration, a process that was often identified with protoplasm and was the focus of speculative accounts by researchers such as Pflüger and Nägeli. As biochemists turned their attention to the oxidation of foodstuffs to carbon dioxide and water, two models competed for attention. Both recognized that

reactions between oxygen and foodstuffs would not occur under ordinary atmospheric conditions and that enzymes must be responsible. The Thunberg-Knoop-Wieland model emphasized reactions in which enzymes operated on a substrate to release hydrogen atoms (which would then combine with molecular oxygen, were it available). The other model, developed by Otto Heinrich Warburg, construed the enzyme (ferment by his terminology) as operating on oxygen, which would then combine with hydrogen from the substrate.

Heinrich Otto Wieland was an organic chemist who began his exploration of oxidation using inorganic catalysts such as palladium black. He found that when no oxygen was present, the catalyst would operate for a short time before it became saturated with hydrogen. He proposed that when oxygen was available, it served only to receive the hydrogen removed from the substrate. He substantiated this proposal by showing that methylene blue, a synthetic dye that is readily reduced to leuco-methylene blue, could substitute for oxygen in maintaining the reaction (Wieland, 1913). He proposed the following schema for oxidation of a compound RH₂ to R (Pd designates palladium, the catalyst, and Mb methylene blue, the dye):

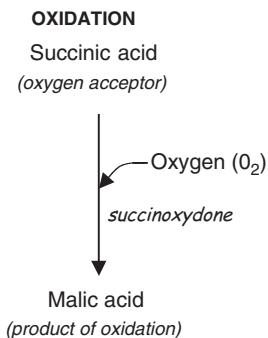


When a substrate such as an aldehyde (RCHO) lacked two removable hydrogen atoms, he proposed that it was first hydrated (a water molecule was added) and then two hydrogen atoms were removed:



Wieland's scheme established a new conceptualization of oxidation as dehydrogenation – the removal of hydrogen rather than the addition of oxygen – and of its relation to reduction. Since the hydrogen released by the substance being oxidized had to be accepted by another substance that was thereby reduced, he proposed that these reactions were necessarily coupled as “two expressions of one process of dehydrogenation” (p. 3340). Wieland then extended this account to biological oxidation. He proposed that oxygen, when present, was the substance reduced in cells, yielding hydrogen peroxide (H₂O₂) that the enzyme catalase would quickly convert to water. Importantly, he showed that when oxygen was not present, oxidation could still occur in biological entities. Specifically, ethanol and acetaldehyde could be oxidized to acetic acid in bacteria if methylene blue was available as a hydrogen acceptor.

(a) Battelli and Stern (1907-14)



(b) Thunberg (1916)

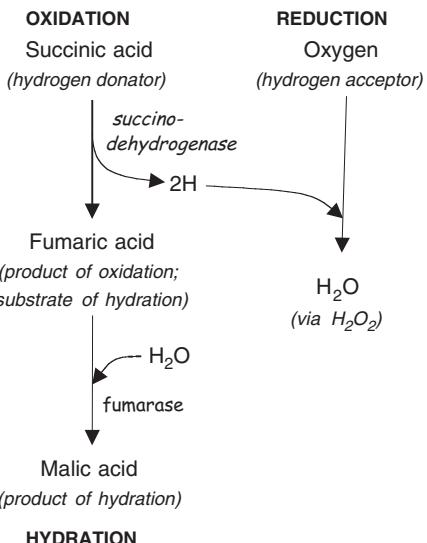
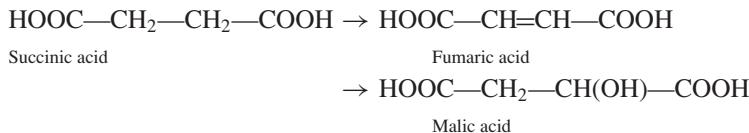


Figure 3.12. Two early accounts of the metabolism of succinic acid to malic acid. They differ in how oxidation is conceptualized and in the number of reactions involved. (a) Batteli and Stern assumed that activated oxygen is added directly to the substrate, succinic acid. (b) Thunberg identified fumaric acid as an intermediate substance between succinic and malic acids; incorporated paired reduction-oxidation reactions, as proposed by Wieland; and posited specific enzymes for which better evidence later emerged. It was also determined later that the carrier coenzyme FAD, not molecular oxygen, was the immediate hydrogen acceptor.

Wieland's proposal received little uptake from biochemists until it was further developed by Thorsten Ludvig Thunberg, who recognized that it could be applied to the experimental results that Federico Battelli and Lina Stern (1911) had obtained concerning the oxidation of succinic acid. Their approach was to add metabolic substrates to suspensions of minced animal tissue presumed to include enzymes of interest, and use manometers to supply oxygen gas and measure how much was absorbed during the resulting oxidation reactions. As illustrated in Figure 3.12(a), they interpreted their findings on succinic acid in terms of an enzyme that *activated* the dissolved oxygen so that it would combine with the substrate (succinic acid) to yield the observed product (malic acid).⁴⁰ Subsequently, however, Hans Einbeck (1914) demonstrated that fumaric acid was created in the process:

⁴⁰ The named the enzyme *succinoxidone*, using an alternative to the traditional ending –ase since this enzyme was not soluble.



As illustrated in Figure 3.12(b), Thunberg recognized that succinic acid was first oxidized (dehydrogenated) by the removal of two hydrogen atoms, yielding fumaric acid, which then gained from water two hydrogen atoms plus an oxygen atom, yielding malic acid. Thus, on this view the additional oxygen found in malic acid came not from molecular oxygen but from the water molecule that was added to fumaric acid. Molecular oxygen did not figure in the oxidation reaction directly but was the recipient of the liberated hydrogen and so reduced to water. On this revised account there were three main reactions: (1) oxidation of succinic acid, (2) hydration of fumaric acid, and (3) reduction of oxygen. To show that oxidation reaction could occur in animal tissue independently of the reduction of oxygen, Thunberg adopted Wieland's strategy of using methylene blue rather than molecular oxygen as the hydrogen acceptor. To conduct these experiments, he created a special device (later known as the *Thunberg tube*) from which all oxygen was removed. When a minced muscle preparation and methylene blue were placed in the tube, nothing happened; however, when succinic acid was added, the methylene blue was reduced, as indicated by its rapidly decolorization (Thunberg, 1916). He subsequently showed that the same was true of various other organic acids (lactic acid, fumaric acid, malic acid, citric acid, etc.): They were dehydrogenated when methylene blue was supplied. Thunberg (1920) determined that reactions involving different substrates were differentially affected by heat, which he took as evidence that different enzymes were responsible for each reaction.

Warburg, in contrast, construed a reaction with oxygen as the key step in oxidation and argued that the *activation* of oxygen, which prepared it to combine with substrates, was the major catalytic event (thus employing the same model as Battelli and Stern).⁴¹ In his earliest work Warburg focused on cell membranes, whose existence had finally been convincingly demonstrated by

⁴¹ Given his focus, Warburg's challenge was to measure the amount of oxygen taken up in a reaction. In his earliest research he used titrimetric methods but during a visit to Joseph Barcroft's laboratory at Cambridge in 1910 he observed the Haldane-Barcroft blood-gas manometer, a device which kept the volume of the gas constant so that pressure would change as gas was added or lost. Warburg modified their instrument to measure rates of gas exchange and used it in most of his subsequent research. The resulting device came to be known as the Warburg manometer and has been widely used in biochemical studies of reactions involving gas exchanges, such as oxidations (Warburg, 1923; Warburg, 1925a).

Ernest Overton (1899). Overton had shown that the membrane, comprised largely of fats and lipids, served as a semipermeable osmotic barrier between a cell and its environment; he also had investigated the ability of hundreds of organic solutes to cross cell membranes. Working with sea urchin eggs, Warburg (1910) pinpointed the membrane as the site of respiration by showing that alkaline solutions increased the respiration of sea urchins without altering the alkalinity of the protoplasm, and that fatty acids and organic solvents, which affected membranes, decreased respiration. Warburg (1913b) subsequently investigated the effect of narcotics such as ethyl urethane on respiration, and from this research eventually concluded that it was not the lipids of the membranes that they affected, but solid particles in the protoplasm which turned out to be mitochondria. Even as the details of his account shifted, a constant theme at this stage in Warburg's career was his opposition to the view that soluble enzymes alone were responsible for significant biological processes – he maintained that they operated in context of structured systems (Warburg, 1913a).⁴²

It was in this context of emphasizing that enzymes operated in structured systems that Warburg first introduced the term *Atmungsferment* to designate the agent responsible for biological respiration. He viewed it as operating on oxygen, activating it so that it would combine with hydrogen in the substrate undergoing oxidation. Working with Meyerhof, Warburg connected the effect of citric acid and tartaric acid in halting respiration in sea urchin eggs to their ability to chelate (form ring structures with) heavy metals. He concluded “that the oxygen respiration in the egg is an iron catalysis; that the oxygen consumed in the respiratory process is taken up initially by dissolved or absorbed ferrous ions” (Warburg, 1914, pp. 253–4, translated in Fruton, 1972, p. 302). Now Warburg proposed that *Atmungsferment* consisted of ferrous iron adsorbed onto the membranes of the cell. Warburg also developed model systems of activated charcoal or pyrolysed (heat-transformed) blood in which to study the reaction.

⁴² Warburg also criticized Buchner's characterization of zymase as a soluble enzyme. He interpreted the slowing of fermentation in cell-free extracts identified by Harden and Young as indicating the destruction of the membrane which was critical to the normal operation of the ferment. At this point, Warburg insisted that both cell structure and enzymes (for him, ferment) were required: “The question always comes to this: cell action or ferment action? Structure action or ferment action? I hope I have demonstrated to you today that there is no dichotomy here at all: both ferment chemists and biologists are right. The acceleration of energy-producing reactions in cells is a ferment action *and* a structure action; it is not that both ferment *and* structure accelerate, but that *structure accelerates ferment action*” (1913a, pp. 20–1, translated in Kohler, 1973, pp. 189–90).

At this point, military service during World War I interrupted Warburg's research. When he resumed it, he characterized *Atmungsferment* more like an enzyme and focused on how it could be inhibited, first by hydrogen cyanide (Warburg, 1925b) and later by carbon monoxide (Warburg, 1929). Although he could not isolate the enzyme, he developed techniques to fingerprint it by its absorption spectrum. Throughout this period, though, he emphasized that the critical operation involved the activation of oxygen. He fiercely opposed Wieland's contention that removal of hydrogen atoms was the crucial step in oxidation and that dehydrogenases operated on the substrate to activate and remove pairs of hydrogen atoms.

The competing theories of Wieland and Warburg each offered a simple mechanism to explain cellular respiration – one operation activated a molecule, which then reacted with the other molecule (removing it from the substrate if necessary). Their alternative accounts became the focus of a bitter controversy as each party maintained that he had identified the critical operation. However, other researchers began to consider the possibility that both processes were involved in cellular respiration. As proposed by Albert Szent-Györgyi (1924), “In cellular oxidation the *activated hydrogen* is burned by the *activated oxygen*. In the terminology of the hydrogen activation theory this means that molecular oxygen is not a hydrogen acceptor; the biological hydrogen acceptor is the oxygen activated in Warburg’s system” (Szent-Györgyi, 1924, p. 196, translated by Fruton, 1972, p. 322). On this proposal, the claims of Wieland and Warburg did not intrinsically conflict but instead, as Figure 3.13 shows in skeletal form, figured at opposite ends of the same pathway.

As it turned out, however, the mechanism was far more complex than one obtained by simply combining the two proposals. Working from both ends of the pathway, researchers in the 1920s and 1930s identified a multitude of operations that linked them into a complex mechanism. (Bechtel and Richardson, 1993, characterized this as a move from *simple localization* to *complex localization*.) Thunberg himself took the first step by proposing the idea of a sequence of dehydrogenation reactions in which the product of a given dehydrogenation was further dehydrogenated (or otherwise operated on) in another reaction.⁴³ In particular, he proposed the following

⁴³ Thunberg actually had the idea of a sequences of reactions as early as 1913, before he encountered Wieland's conception of dehydrogenation: “The oxidative processes in the living cell must be thought of as forming chain reactions, a series of reactions connected to one another in such a way that, by and large, none of the links in the reaction chain can proceed more rapidly than the others” (1913, translated in Holmes, 1986, p. 68).

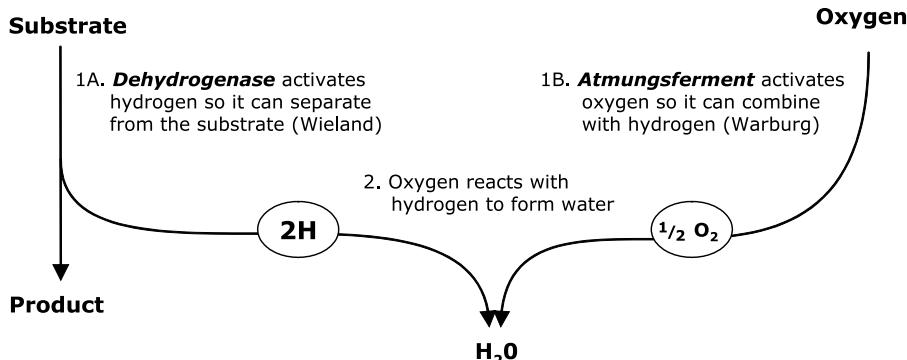
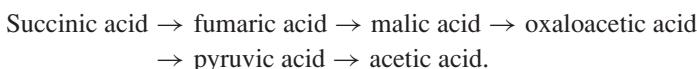
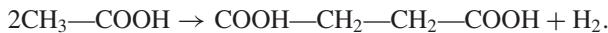


Figure 3.13. Schematic account of how Wieland's account of respiration in terms of dehydrogenation could be linked to Warburg's proposal of an enzyme acting on molecular oxygen.

reaction pathway:



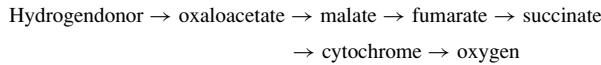
He then faced a problem in specifying what happened next – it was not possible to remove two hydrogen atoms from acetic acid. In response to this problem, Thunberg offered a bold proposal – he proposed “a reaction in which two acetate molecules are simultaneously each deprived of one hydrogen atom, with the joining of their carbon chains into one. The substance which must therefore form is succinic acid” (1920, passage translated by Holmes, 1986, p. 69). The reaction Thunberg proposed was the following:



In effect, Thunberg had proposed the cycle of reactions illustrated Figure 3.14.⁴⁴

Fifteen years later Hans Krebs, together with William Johnson, incorporated the core of this idea into the citric acid cycle (also known as the *tricarboxylic acid cycle* and later as the *Krebs cycle*). Instead of two molecules of

⁴⁴ A related idea was advanced by Albert Szent-Györgyi (1937) on the basis of his studies adding different four-carbon dicarboxylic acids to suspensions of minced pigeon-breast muscle. He proposed a scheme in which the four-carbon acids are viewed as performing hydrogen transport, not steps in oxidation of carbohydrate:



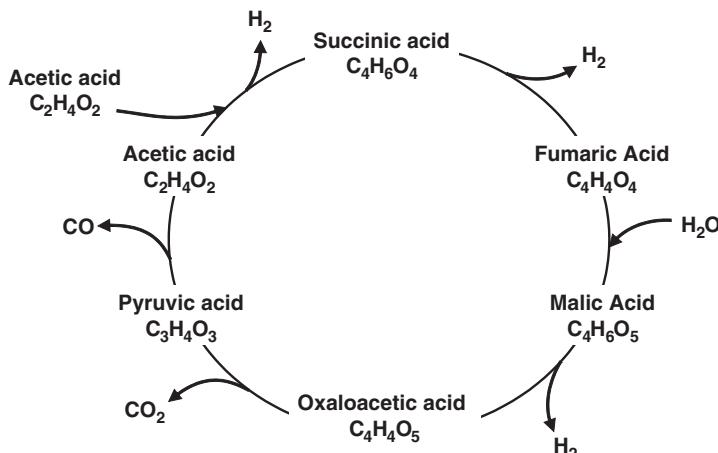


Figure 3.14. Cycle of reactions proposed by Thunberg.

citric acid combining to form succinic acid, Krebs proposed that oxaloacetic acid combined with a three-carbon substance that Krebs temporarily called *triose* to generate citric acid, with the citric acid then undergoing a sequence of reactions resulting in succinic acid (Krebs & Johnson, 1937). (See center part of Figure 3.15.) It was soon realized that pyruvic acid (pyruvate), a product at the end of glycolysis, provided the material that would react with oxaloacetic acid. Figuring out the exact linkage provided a bit of a challenge. After Fritz Lippman (1945) discovered coenzyme A, evidence began to develop that it figured in the connection. Feodor Lynen initiated research attempting to show that acetic acid figured in the pathway between pyruvic acid and citric acid, but because ordinary acetic acid would not condense with oxalacetic acid to create citric acid, he speculated that *activated acetic acid* must be involved. Lynen and Richert (1951) demonstrated that the activated acetic acid was a thio (sulfur) ester of acetylated coenzyme A, a compound now known as acetyl-CoA. With this account of the connection, the citric acid cycle was linked to the pathway of glycolysis (as well as the pathways of fatty acid metabolism and protein metabolism).

Another key advance, working from the oxygen end of the overall process, came from a very unlikely source. David Keilin was studying the respiration of the parasite horse bot-fly (*Gasterophilus intestinalis*), when he detected a disappearance of hemoglobin in later stages of metamorphosis from the pupa to adult fly stage. Spectroscopic examination of flies that died in captivity revealed a pigment with four distinct absorption bands. Keilin considered the possibility that the pigment originated from the larval hemoglobin,

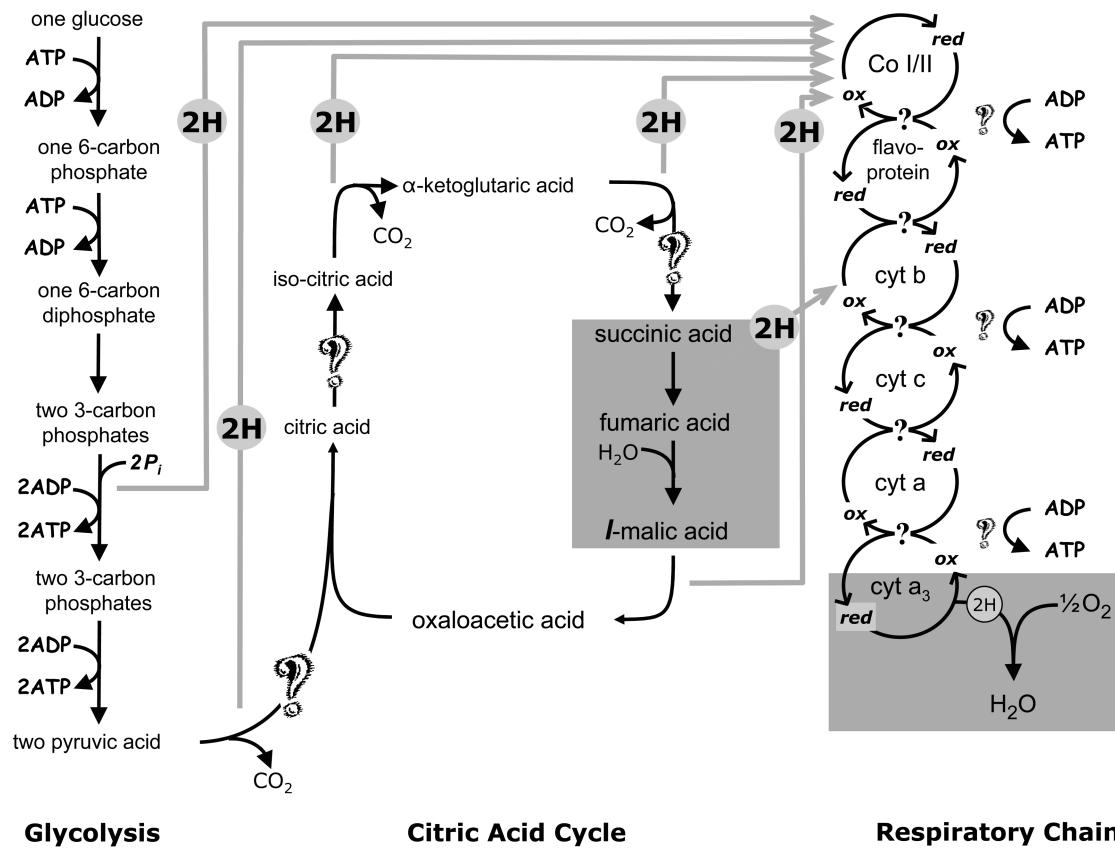


Figure 3.15. Overview of cellular respiration as it was understood in the early 1940s. Question marks indicate points at which researchers realized there were steps they did not understand.

but ruled that out. He then began to look in a wide range of other species and continued to find the same four-banded absorption spectrum. While examining a yeast preparation, he observed that immediately after shaking it, he failed to find the absorption pattern. Yet, “before I had time to remove the suspension from the field of vision of the microspectroscope, the four absorption bands suddenly reappeared” (Keilin, 1966, p. 145). Searching the published literature, Keilin discovered that Charles MacMunn had made the same finding in the 1880s (MacMunn, 1884; MacMunn, 1886). Keilin (1925) went beyond MacMunn’s observation in determining that these bands came from three different hemochromogens which he labeled *cytochrome a, b, and c*. Each cytochrome itself was composed of at least one protein with an iron-porphyrin prosthetic group, with the iron atoms accounting for the reversible reactions. By 1939 Keilin, collaborating with Edward Hartree, had distinguished *cytochrome a₃* from *a* and characterized the four cytochromes as forming a catalytic chain “which, by utilizing molecular oxygen, can easily oxidize to water certain hydrogen atoms in the substrate molecules activated by specific dehydrogenase systems” (Keilin and Hartree, 1939, p. 190). They named the enzyme catalyzing this oxidation *cytochrome oxidase*. Based in part on their identical absorption spectra, Keilin and Hartree identified cytochrome oxidase with Warburg’s *Atmungsferment*. They also tentatively identified it with *cytochrome a₃* in particular, but commented that the *b-c-a-a₃* system could be regarded as either three hydrogen carriers plus an enzyme or as a four-component chain of enzymes (Keilin & Hartree, 1939). In the 1940s, this reaction sequence was referred to as the *respiratory chain*. Later, the term *electron transport chain* became favored as biochemists discovered additional components and also wished to emphasize that it was pairs of electrons – dissociated from the protons of the hydrogen atom – that were transported down an energy gradient to molecular oxygen.

In his 1932 Nobel lecture Warburg (1932) insisted that *Atmungsferment* was *the* active agent and resisted the claim that it was the cytochromes that were oxidized by activated oxygen. Nonetheless, during this period Warburg made two critical discoveries that filled in the steps between oxidation (dehydrogenation) of substrates and the oxidation-reduction reactions of cytochromes in the electron transport chain. I noted previously that in the mid-1930s Warburg and Christian discovered two coenzymes, which are now called NAD and NADP. In addition, they established that both NAD and NADP figure in connecting the oxidative reactions in the citric acid cycle to the electron transport chain. It took a number of years, though, for investigators to work out which of these two coenzymes participated in which reactions.

In the same period Warburg and Christian (1932) had also identified a “yellow ferment” which they characterized as “oxygen-transporting.” The prosthetic group (coenzyme) turned out to be flavin mononucleotide (FMN). Warburg and Christian (1938) then identified yet another yellow enzyme whose coenzyme, flavin adenine dinucleotide (FAD), consists of FMN plus adenyllic acid. These coenzymes are closely related to vitamin B₂, the vitamin whose deficiency gives rise to pellagra. As with coenzymes I and II, there was considerable confusion for a number of years as to their exact functions and which was the prosthetic group of which flavoprotein. Investigators focusing on the electron transport chain in the 1940s posited two different pathways converging at cytochrome *b*. One coupled the oxidation of most citric acid cycle substrates to the reduction of NAD ($\text{NAD}^+ \rightarrow \text{NADH}$) and, in turn, its oxidation to the reduction of a flavoprotein. The other pathway, based on the fact that succinic acid was the only citric acid cycle intermediate whose oxidation did not produce NADH, coupled the oxidation of succinic acid directly to the reduction of a flavoprotein. It was thought that each of these flavoproteins was then oxidized, coupled with the reduction of cytochrome *b*. Eventually it was determined that FMN was the flavoprotein on the pathway from NADH to cytochrome *b*, whereas FAD played a previously-unsuspected role in the second pathway: the oxidation of succinic acid was coupled to the reduction of FAD, which carried the electrons released by that oxidation into the electron transport chain. Finally, the isolation of ubiquinone, coenzyme Q, from beef heart mitochondria (Crane et al., 1957) led to the recognition that it is an additional substance undergoing reversible oxidation-reduction between both FAD and FMN and cytochrome *b* (see Chapter 6).

With the discovery of the steps involved in the citric acid cycle and the electron transport chain, the oxidation pathway from pyruvic acid to water was complete. One aspect of the process, however, was still not addressed – how the energy released in the oxidative reactions was captured in ATP. Hermann Kalckar (1939) and Fritz Lipmann (1939) both demonstrated that the oxidation of intermediaries such as succinate, malate, and pyruvic acid was accompanied by creation of ATP. Because different substrates all resulted in synthesis of ATP, this suggested that the synthesis occurred in conjunction with the oxidation reactions along the respiratory chain linking dehydrogenation of the substrate with molecular oxygen. Such ATP synthesis came to be known as *oxidative phosphorylation* to contrast it with the phosphorylation of ADP directly linked with metabolic intermediates, a process that was referred to as *substrate phosphorylation*. Both Ochoa (1940) and Belitzer and Tsibakowa (1939) showed that in oxidative phosphorylation more than one ATP molecule was formed per atom of oxygen reduced. By focusing on

phosphate consumed per molecule of lactate oxidized in cat heart extracts, Ochoa argued that in fact three molecules of ATP were formed per oxygen atom reduced (Ochoa, 1943). This P:O ratio was eventually accepted after considerable conflict.

The research on cellular respiration had been remarkably fruitful in the earlier decades of the twentieth century, with major advances achieved in the 1930s. Researchers had figured out the basic schema of the overall process, but also recognized many gaps in their knowledge. Figure 3.15 shows the understanding of the component mechanisms of glycolysis, the citric acid cycle, and electron transport as they were understood in the early 1940s, with question marks indicating points at which investigators recognized important gaps that still needed to be filled in.

The State of Biochemistry circa 1940

Biochemistry not only came into its own in the first four decades of the twentieth century but made impressive advances in understanding chemical processes in the cell. The Embden-Meyerhof pathway provided a comprehensive mechanism for glycolysis, and together the citric acid cycle and electron transport chain provided a very detailed sketch of the mechanism of aerobic cellular respiration. However, two problems were emerging for filling this in. First, researchers faced a challenge in explaining the linkage between the oxidation operations and phosphorylation. Based on the example of fermentation, they assumed that intermediate phosphorylated compounds formed along the electron transport chain that would transfer a high-energy phosphate bond to ADP. As I will discuss in Chapter 6, such compounds were never found. Second, biochemists were unable to carry out the entire reaction in a cell-free extract, a critical step if they were going to satisfy the biochemical standards for successful understanding of a biochemical reaction. That required isolation of the individual components (enzymes, cofactors, etc.) and resynthesis of a functioning system from them.⁴⁵ The problem, as Keilin and Hartree (1940) discovered, was that membranes seemed to be required in any preparation that carried out oxidative phosphorylation. The recognition of the role of membranes in oxidative phosphorylation was important, because

⁴⁵ Twenty-five years later Efraim Racker notes the failure to satisfy this demand as a problem facing the biochemistry of oxidative phosphorylation: “The mechanism of energy production in mitochondria has long defied analysis, since a complex chemical pathway in a living organism cannot really be understood until its intermediate products have been identified and the enzymes that catalyze each step of the process have been individually resolved as soluble components” (1968, p. 32).

it pointed to the need for a bridge to a study of cell structures. Making this connection, however, required appropriate techniques to study cell structures at the appropriate level of organization, which were only in the process of being developed.

3. THE NEED TO ENTER THE TERRA INCOGNITA BETWEEN CYTOLOGY AND BIOCHEMISTRY

In this chapter, I have focused on what cytology and biochemistry were able to contribute to an understanding of how cells performed several important functions of life prior to 1940. After a promising start in the late nineteenth century, cytology stalled in the twentieth century. The chief problem was that cytologists' main tool, the light microscope, could not reveal the details of the internal structures in cell cytoplasm. Beyond the rather crude attempts to correlate structures with activities cells were performing, cytologists on their own could not address the function of these structures. Working at a lower level of organization, biochemists were making great progress in unraveling chemical mechanisms involved in cell processes such as fermentation and cellular respiration. Their main strategy for securing preparations for their investigations, homogenation, destroyed any cell structure, making contract with cytological research difficult.

By 1940, several researchers from both cytology and biochemistry recognized the potential for linking their inquiries. The first edition of Geoffrey Bourne's *Cytology and Cell Physiology* in 1942 bears witness to this desire. Nonetheless, there remained unexplored territory between reconstituted chemical pathways and what could be observed through the light microscope about the structure and behavior of cells. There had been pioneering efforts, especially in the developing fields of cytochemistry and histochemistry, that gave hope of localizing processes within cells and understanding just how structure and function related. But truly productive exploration of this territory required new tools. Two tools that were to be immensely important in exploring this territory were being applied to cells for the first time around 1940 – the ultracentrifuge and the electron microscope. Each gave rise to a problem already noted several times in this chapter: Were the results obtained with the new instruments providing real information about cells or were they merely producing artifacts? This is the topic of the next chapter.